Proceedings of the Summer Meeting of the Crustacean Society and the Latin American Association of Carcinology, Costa Rica, July 2013

Edited by

Ingo S. Wehrtmann and Raymond T. Bauer

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Preface for Zookeys special issue for Proceedings of TCS-ALCARCNUS 2013 Meeting

Costa Rica, a Central American country bordered by the coasts of two oceans and divided by mountain ranges into a mosaic of habitats, is rightly recognized as a hotbed of biological diversity. What an appropriate locale to celebrate the diversity in taxonomy, phylogeny, reproduction, ecology, behavior and a host of other topics on marine, freshwater, and terrestrial Crustacea! Accordingly, carcinologists from around the world met in San José, Costa Rica, in July 2013, under the auspices of The Crustacean Society (TCS) and the Asociación Latinoamericana de Carcinología (ALCARCINUS), to present the results of their research. A total of 175 colleagues from 36 countries attended the meeting, and presented 116 talks and 118 posters.

The idea of publishing the original research of selected presentations in a journal as a meeting proceedings special number was conceived and organized by one of us (IW), who invited RB to share duties with him as a guest editor for this issue. Zookeys, as an open access journal for biodiversity research, was chosen as an appropriate venue for the publication of research on crustaceans presented at the meeting. Conference participants were invited to submit manuscripts they presented at the meeting, and after extensive review and editorial scrutiny, a final total of 22 contributions involving 66 authors from 11 countries were accepted, covering a number of taxa and a variety of topics such as biodiversity, phylogeny, reproductive ecology, biogeography, population biology, and parasitism. We thank the authors, reviewers and able editorial staff of Zookeys for making this special issue on crustacean diversity and biology possible. A very special and sincere thanks goes to Yordanka Banalieva, Editorial Secretar of Pensoft Publishers, who patiently assisted and supported the entire review and editing process for this special volume of ZooKeys. Yordanka, it was a pleasure working with you! Kimberly García helped one of us (ISW) to revise all the editorial details in the manuscripts, which is greatly appreciated. Muchas gracias!

One of the principal purposes of the Costa Rica 2013 – meeting was to bring together crustacean researchers from all over the world and to offer them a platform to present their scientific work and to establish future collaborations. Therefore, we trust that the contributions contained in this proceedings volume of ZooKeys will stimulate further interest and collaborative research on a variety of crustacean taxa.

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RESEARCH ARTICLE

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Genetic utility of natural history museum specimens: endangered fairy shrimp (Branchiopoda, Anostraca)

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Abstract

We examined the potential utility of museum specimens as a source for genetic analysis of fairy shrimp. Because of loss of their vernal pool habitat, some fairy shrimp (including *Branchinecta sandiegonensis* and *B. lynchi*) are listed as threatened or endangered in Southern California by the United States Fish and Wildlife Service. Management of those species requires extensive population genetics studies and the resolution of important genetic complexity (*e.g.* possible hybridization between endangered and non-endangered species). Regulations mandating deposition of specimens of listed species have resulted in thousands of specimens accessioned into the Natural History Museum of Los Angeles County that have been preserved in a variety of solutions. We subsampled those specimens, as well as other Anostraca with known collection and preservation histories, to test their potential for genetic analysis by attempting DNA extraction and amplification for mt16SrDNA. Fixation and preservation in not denatured ethanol had a far greater sequencing success rate than other (and unknown) fixatives and preservatives. To maximize scientific value we recommend field preservation in 95% not denatured ethanol (or, if pure ethanol is unavailable, high-proof drinking spirits, *e.g.* Everclear™, or 151 proof white rum), followed by storage in 95% not denatured ethanol.

Keywords

Museum specimens, Anostraca, Branchinectidae, *Branchinecta sandiegonensis*, *B. lynchi*, *B. lindahli*, endangered, threatened, vernal pool, California

Introduction

The largest collection of endangered Southern Californian fairy shrimp in the United States of America is at the Natural History Museum of Los Angeles County (LACM). The LACM is working closely with the United States Fish and Wildlife Service (USFWS) to increase the scientific value of these specimens for both morphological and molecular studies. Fairy shrimp occur in ephemeral vernal pool habitats worldwide (Keeley and Zedler 1998). In densely human populated areas, their fragile habitats continue to be severely degraded and many have been destroyed by urbanization (Bauder and McMillan 1998, King 1998, Simovich et al. 2013).

At least 15 plant species are recognized as threatened or endangered in California vernal pool habitats, but only a few invertebrates are similarly recognized (USFWS 2005). *Branchinecta conservatio*, *B. longiantenna*, and *B. sandiegonensis* are listed as "Endangered", and *B. lynchi* is listed as "Threatened" by the USFWS. In California, the USFWS issues permits for the collection of fairy shrimp and requires the deposition of endangered and threatened species in one of two repositories: the LACM or the California Academy of Sciences in San Francisco. Traditionally, Southern California specimens come to the LACM and northern California collections go to the California Academy. Since 1995 about 5,000 lots of *B. lindahli*, *B. lynchi*, and *B. sandiegonensis* have been accessioned into the LACM collections. This represents about 95% of our total anostracan holdings.

Simovich et al. (2013) suggest that human disturbance is increasing the generalist *B. lindahli*'s range, which in turn is eroding the native range of *B. sandiegonensis*. Due to increasing sympatric distribution of these species, these authors (and Fugate 1998 before them) claim that the endangered and non-endangered species (*B. sandiegonensis* and *B. lindahli*) are hybridizing, thereby threatening the genetic integrity and persistence of *B. sandiegonensis*. Using a PCR-based screen using mitochondrial DNA to determine maternal lineage, in conjunction with morphological examination, Simovich et al. (2013) claim putative hybrids share their maternal DNA with the more common species at a site. Unfortunately, their claims are not testable or reproducible as the specimens used in their study are unavailable. Aside from this study, only an unpublished master's thesis exists that addresses genetic aspects of putatively hybrid populations of Southern California *Branchinecta sandiegonensis* (Andrews 2013). That study depended on prior researchers' assessments of hybridization in individual pools. These claims of hybridization underscore the need for comprehensive molecular studies to characterize the actual genetic diversity and species boundaries of Southern California fairy shrimp before further management and remediation recommendations are made.

In contrast to the lack of work being conducted on endangered Southern Californian fairy shrimp, there has been a large amount of work studying the genetics and phylogeographics of the endangered Californian salamander *Ambystoma tigrinum* (Amphibia: Caudata: Ambystomatidae) (Ryan et al. 2009, Johnson et al. 2010, Johnson et al. 2011). These studies were made possible in large part by a very extensive collection of samples — tail clippings — of *A. tigrinum* that span the salamander's geographic range through the last 25 years. Just as important as the breadth of the collection of tail clippings was that these samples were preserved with a method that made them accessible for molecular study decades later. The findings from these studies have already helped the management of *A. tigrinum* by identifying which populations have the greatest genetic diversity and allowing USFWS to target high value populations for increased protection (Johnson et al. 2011). The LACM is working closely with USFWS to assemble a collection of endangered Californian fairy shrimp necessary for similar genetic and phylogeographic studies. Both the LACM and USFWS fully expect that one day such studies will help better inform and shape the management of endangered fairy shrimp.

In this study we test whether preservation in pure not denatured ethanol makes anostracan museum specimens more readily accessible for molecular studies over anostracan museum specimens that had historically been fixed in denatured ethanol, isopropyl, or even acetone, then transferred into pure not denatured ethanol. Our study compares the success rates of amplifying a fragment of mt16SrDNA for specimens preserved in not denatured ethanol and for specimens in other preservatives. Because of their rarity and the difficulty in collecting fresh fairy shrimp specimens, being able to use specimens already in museum collections would be advantageous. To improve the utility of future collections, we suggest improvements in field and post-field preservation and handling based on our findings. If adopted, these improvements will greatly enhance the genetic usefulness of specimens and thereby allow more thorough assessments.

Methods

Material examined

We first inventoried, digitized, and georeferenced our entire anostracan collection — approximately 5,000 lots. We selected 50 specimens from across the taxonomic range that had been contributed by different collectors and consulting companies using a range of different field preservatives prior to deposition at the LACM (at the LACM, all specimens are transferred from the field preservative into fresh museum-grade not denatured ethanol). We then attempted to amplify a ~550 bp mt16SrDNA fragment (see Table 1).

DNA extractions

The starting material for DNA extractions varied among samples, one thoracopod to an entire animal, depending on total animal body size. Tissue samples were placed on paper towel to dry. Precipitation Reagent (Epicentre MMP03750) was added to each sample and vortexed vigorously for 10 sec., then centrifuged at 4 °C for 10 min. at 14,000 rpm. The supernatant (~300 µl) was transferred to a 2 ml tube. Genomic DNA

Table 1. Extractions and amplifications attempted for this publication. Taxa arranged in alphabethical order. Locality, specimen collection date, collector, and preservative are as transcribed from specimen labels. Specimen condition and body part used in extraction are indicated if this information was recorded. Double-stranded DNA concentration in ng/µL. Qubit value indicated as low, *i.e.*, 0<0.05 ng/µL. Asterisk (*) indicates sequence was generated and is listed in Table 2.

	Taxon	Date of collection	Description of preservative on label	Locality	Collector	Part of specimen used	Extraction number	Outcome	dsDNA ng/µL 0<0.05
1	*Artemia monica*	06-Jul-90	70% ethanol	California, Mono County, Mono Lake, south Tufa Reserve	H. Kuck	1 broken specimen	2013	contaminated; blasts as *Homo*	0
2	*Artemia monica*	01-Jan-10	fixed and preserved in 95% ethanol	California, Mono County, Mono Lake	M. Hauser	1 whole squished specimen	2008	*beautiful sequence	39.1
3	*Branchinecta coloradensis*	23-Apr-92	70% ethanol	California, Lassen County, Hog Flat Reservoir	King, Gluesenkamp, Tritt	1 broken specimen	2003	failed	0
4	*Branchinecta dissimilis*	23-Mar-92	70% ethanol	California, Shasta County, Fall River	King, Gluesenkamp, Kloock	2 broken pieces	2017	failed	0.2
5	*Branchinecta gigas*	unknown	acetone	California, San Bernadino County, Mojave Desert	J. Martin, J. Plum	2 phyllopods only	1990	failed	0.17
6	*Branchinecta gigas*	unknown	not indicated	Washington, Grant County	unknown	1 small whole specimen	2006	failed	0.13
7	*Branchinecta gigas*	unknown	not indicated	Washington, Grant County	unknown	dissected off egg sack with eggs	2007	failed	0
8	*Branchinecta lindahli*	27-Dec-12	fixed and preserved in 95% ethanol	California, San Diego County, Marine Corps Base Camp Pendleton	L. Woolley	not recorded	2036	failed	no data
9	*Branchinecta lindahli*	27-Dec-12	fixed and preserved in 95% ethanol	California, San Diego County, Marine Corps Base Camp Pendleton	A. Fisher	not recorded	2037	failed	no data
10	*Branchinecta lindahli*	29-Dec-12	fixed and preserved in 95% ethanol	California, San Diego County, Marine Corps Base Camp Pendleton	A. Fisher	not recorded	2038	failed	no data
11	*Branchinecta lindahli*	28-Dec-11	fixed and preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook, et al.	egg sac only	1992	*beautiful sequence	6.62

	Taxon	Date of collection	Description of preservative on label	Locality	Collector	Part of specimen used	Extraction number	Outcome	dsDNA ng/µL 0<0.05
12	*Branchinecta lindahli*	02-Apr-12	fixed and preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook	1 gravid female	2026	*beautiful sequence	13
13	*Branchinecta lindahli*	02-Apr-12	preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook	1 gravid female	2027	*beautiful sequence	11.3
14	*Branchinecta lindahli*	02-Apr-12	preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook	1 gravid female	2034	failed	0
15	*Branchinecta lindahli*	02-Apr-12	preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook	1 squished male	2028	*beautiful sequence	29
16	*Branchinecta longiantenna*	23-Mar-10	preserved in 70% ethanol	California, San Luis Obispo County, California Valley	Chris Powers	posterior half of single broken specimen	2005	failed	39.6
17	*Branchinecta lynchi*	27-Feb-01	fixed and preserved in 95% ethanol	California, San Luis Obispo County, Paso Robles	M. Dallas	1 specimen, not gravid, not obviously male	2032	contaminated; blasts as cladoceran	18.7
18	*Branchinecta lynchi*	13-Jan-04	fixed and preserved in 95% ethanol	California, Santa Barbara Co., Los Padres National Forest	T. Murphey	squished gravid female	2030	failed	0.3
19	*Branchinecta lynchi*	03-Feb-05	fixed and preserved in 95% ethanol	California, San Luis Obispo County	D. Hacker	posterior half of gravid female	2033	failed	51.4
20	*Branchinecta lynchi*	17-Feb-05	fixed and preserved in 95% ethanol	California, Santa Barbara Co., Los Padres National Forest, Branch Mountain Quad	T. Murphey	squished gravid female; all animals in this lot are pretty mangled	2031	failed	17
21	*Branchinecta mackini*	unknown	70% ethanol	Washington, Grant County	unknown	1 specimen	1991	failed	0.254
22	*Branchinecta mackini*	03-Apr-93	70% ethanol	California, San Bernadino County, Mojave Desert	C. Cash-Clark, T. Clark	1 specimen	2019	failed	0.16

	Taxon	Date of collection	Description of preservative on label	Locality	Collector	Part of specimen used	Extraction number	Outcome	dsDNA ng/μL $0<0.05$
23	*Branchinecta orientalis*	22-Aug-02	95% ethanol	Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay)	R. Wetzer, S.L. Boyce, N.D. Pentcheff	1 whole small specimen	2004	failed	24.4
24	*Branchinecta sandiegonensis*	09-Mar-05	preserved in 70% denatured ethanol, transferred to 70% ethanol	Mexico, Baja California, Tijuana, Jesus Maria Mesa	K.B. Clark	1 gravid female	2024	failed	0.7
25	*Branchinecta sandiegonensis*	13-Jan-11	preserved in 70% denatured ethyl alcohol, transferred to 70% ethanol	California, San Diego County, Brown Field Municipal Airport	D. Wolff	posterior half of gravid female	2029	failed	0
26	*Branchinecta sandiegonensis*	24-Nov-08	preserved in 95% ethanol	California, San Diego County, Ramona Water District, Ramona Spray Fields	E. Ervin	eggsac + furca from female	2023	failed	4.7
27	*Branchinecta sandiegonensis*	28-Dec-11	preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook, et al.	anterior portion of female specimen	1995	failed	25.7
28	*Branchinecta sandiegonensis*	17-Dec-07	transferred to 95% ethanol Feb. 2011	California, San Diego County, Otay Mesa, Dexstar Property	C. Powers	1 male	2025	failed	49.2
29	*Branchinecta*	28-Dec-11	preserved in 95% ethanol	California, San Diego County, San Diego, Carmel Mountain Preserve	J. Snapp-Cook, et al.	1 specimen	1993	failed	7.5
30	*Branchipodopsis affinis*	22-Aug-02	95% ethanol	Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay)	R. Wetzer, S.L. Boyce, N.D. Pentcheff	1 small specimen	2001	failed	18.8
31	*Branchipodopsis affinis*	22-Aug-02	95% ethanol	Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay)	R. Wetzer, S.L. Boyce, N.D. Pentcheff	1 small specimen	2002	failed	49

	Taxon	Date of collection	Description of preservative on label	Locality	Collector	Part of specimen used	Extraction number	Outcome	dsDNA ng/µL 0<0.05
32	*Chirocephalus*	22-Aug-02	95% ethanol	Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay)	R. Wetzer, S.L. Boyce, N.D. Pentcheff	1 whole squished animal	2018	*beautiful sequence	57.9
33	*Eubranchipus holmanii*	07-May-40	70% ethanol	Canada, Nova Scotia, Edinberg [sic]	D. Belk	1 male specimen	2015	contaminated; blasts as *Homo*	0
34	*Eubranchipus*	01-Apr-32	70% ethanol	Canada, Ontario, Saint Thomas	M.S. Ferguson	anterior end of broken specimen	2014	failed	0
35	*Eubranchipus*	30-Apr-99	70% ethanol	Minnesota, Bloomington	A.B. Forbes	1 female – doesn't look well preserved	2022	failed	0
36	*Eubranchipus*	15-May-12	fixed and preserved in 95% ethanol	California, Lassen County, Poison Lake	M. Hauser, D. Striley	posterior half of the single mushy specimen	2020	*beautiful sequence	27.3
37	*Linderiella occidentalis*	19-Feb-92	70% ethanol	California, Tehama County, Tuscan Buttes	King, Mazzucco, Scuderi	2 pieces broken specimen	2000	contaminated; blasts as *Homo*	0.14
38	*Linderiella occidentalis*	24-Mar-92	fixative unknown – transferred to 70% ethanol	California, Tehama County, Dale's Plains, Dale's Lake	King, Gluesenkamp, Kloock	1 whole specimen	1987	failed	0.225
39	*Linderiella santarosae*	26-Mar-04	70% ethanol	California, Riverside County, Murrieta, Mesa de Colorado, Santa Rosa Plateau	M. Angelos	1 small female specimen	1999	contaminated; blasts as *Homo*	0.293
40	not identified	08-Jun-11	fixed and preserved in 95% ethanol	Utah, Wallsburg, near Provo-Jordan River Pkwy	M. Hauser	1 female specimen	2021	failed	47.6
40	*Phallocryptus*	22-Aug-02	95% ethanol	Mongolia, Dundgovi' aimag, northwest of Delgerhangay (Khashaat/Delger Khanay Uul)	R. Wetzer, S.L. Boyce, N.D. Pentcheff	posterior half of adult specimen	2009	*beautiful sequence	10.3
42	*Pristicephalus comptus*	13-Apr-36	70% ethanol	Tennessee, Reelfoot Lake	unknown	1 specimen, this lot had previously dried and had been realcoholed	2012	failed	0

	Taxon	Date of collection	Description of preservative on label	Locality	Collector	Part of specimen used	Extraction number	Outcome	dsDNA ng/μL $0<0.05$
43	*Streptocephalus sealli*	15-Aug-55	70% ethanol	California, Tulare County, Yosemite, Tioga Pass	unknown	1 specimen, these had been previously dried and realcoholed	1998	failed	0.213
44	*Streptocephalus sealli*	15-Aug-55	95% ethanol	California, Mariposa County, Yosemite, May Lake Trail	unknown	posterior end of animal	1989	failed	0.18
45	*Streptocephalus texanus*	27-Aug-56	70% ethanol	New Mexico, Cain Ranch	S.F. Wood	dissected egg sack	2010	failed	0
46	*Streptocephalus woottoni*	30-Mar-06	70% ethanol	California, San Diego County, Camp Pendleton, Marine Corps Base	S. Baldwin	~5 phyllopods dissected off single specimen (only 1 specimen in the lot)	1994	failed	7.28
47	*Streptocephalus woottoni*	01-Apr-05	not recorded	California, San Diego County, Carlsbad, Poinsettia Lane Commuter Station Vernal Pools	J. Snapp-Cook	posterior half of male (already broken)	2016	*good sequence	16
48	*Streptocephalus woottoni*	29-Jan-03	preserved in 70% ethanol	California, Riverside County, Temecula	unknown	3-4 phyllopods removed from single specimen	1997	failed	6.8
49	*Tanymastix stagnalis*	12-Aug-34	70% ethanol	Denmark, Raabjerg Mile	E.W. Kaiser	3 broken pieces used	1996	failed	0.224
50	*Thamnocephalus platyurus*	01-Aug-56	70% ethanol	New Mexico, Gran Quivira	S.F. Wood	posterior portion	2011	failed	0

was extracted and purified with a Quick-gDNA™ MiniPrep Kit (Zymo Research) following the manufacturer's instructions, and eluted in a final volume of 60 µl of distilled water (in two elutions of 30 µl). Double-stranded DNA concentration of extractions was quantified using a Qubit 1.0 Fluorometer (Life Technologies) (see Table 1).

PCR protocols

The mt16SrDNA fragment was amplified with universal 16Sar and 16Sbr primers (Palumbi et al. 1991) and both strands were sequenced. PCR reactions were done in a final volume of 50 µl. The volume of DNA used in each reaction varied from 2–25 µl depending on the DNA concentration measured on the Qubit. When possible, we tried to use at least 50 ng of DNA. Two different PCR reaction setups were used, as some samples successfully amplified with one, but not with the other. The first setup consisted of 10 µl of GoTaq Promega Buffer 5x, 5 µl of 2.5 mM $MgCl_2$, 4 µl of a 10 mM dNTP mixture, 2 µl of each primer at 20 µM, and 0.3–0.5 µl of GoTaq Polymerase at 5 U/µl (Promega). The second setup consisted of 25 µl of a 2x PCR Master Mix with 1.5 mM $MgCl_2$ (Thermo Scientific), and 1 µl of each primer at 20 µM. Both positive and negative controls were run in each experiment. Amplifications were performed in a BIO-RAD S1000 Thermal Cycler, with the following thermocycler conditions: an initial step of 5 min. at 95 °C, 35 cycles of 30 sec. at 95 °C, 30 sec. at 48 °C, 45 sec. at 72 °C, and a final extension of 10 min. at 72 °C. Amplifications were checked by running 5 µl of the PCR product on a 1.5% agarose gel. All failed amplifications were retried at least twice with different polymerases, buffers, and $MgCl_2$ concentrations. Successful PCR reactions were then purified with a DNA Clean and Concentrator-5 Kit (Zymo Research) and sequenced with both primers at Laragen Inc, Culver City, CA. Chromatograms were visually inspected and edited with 4Peaks (Griekspoor and Groothuis 2014).

Contamination screening

Sequences were edited and contigs assembled in the software program Sequencher (Gene Codes Corporation 2004), and all contigs were BLAST searched in the NCBI database to verify they were not contaminants (*i.e.*, that sequence was indeed from the taxon of interest).

Statistical testing

A Fisher's exact test (two-tailed, $\alpha=0.05$) was used to determine whether there was a statistically significant difference in sequencing success between the ethanol-preserved and other samples (Zar 1999). A Qubit 1.0 Fluorometer (Life Technologies) was used to quantify double-stranded DNA (Table 1). A one-tailed Mann-Whitney U test (Zar 1999) was used to assess statistical significance between double-stranded DNA concentration and amplification success.

Results

Of the 50 individual anostracan samples on which we attempted PCR amplification, 13 were known to have been fixed and preserved in pure 95% ethanol, and 37 samples had unknown preservation histories but were suspected of being fixed and stored in denatured ethanol sometimes for years, until they were incorporated into the LACM collection. Of the samples fixed and preserved in 95% ethanol, 62% (8 out of 13) yielded useable mt16SrDNA sequences. In contrast, of the samples with unknown fixative and preservative history, only 3% (1 out of 37) yielded useable mt16SrDNA. The nine sequences generated here are available on GenBank (see Table 2). Sequencing success between samples fixed and preserved in ethanol and other samples was significantly different (Fisher's exact test, two-tailed, $P < 0.0007$).

The one-tailed Mann-Whitney U Test showed that there was a difference (at the $\alpha = 0.05$ level) between Qubit measurements of double-stranded DNA concentration for successful sequences vs. failed sequences, when amplifications of contaminants were considered as failed amplifications. However, direct examination of the data (see Table 1) showed that DNA concentration was a very poor predictor of sequencing success (except for the case of 0 or near-0 readings, which invariably failed).

Discussion

Existing museum specimens

Specimens known to be collected and preserved in 95% ethanol were successfully extracted, amplified and sequenced at a much higher success rate than those with unknown preservation history (probably denatured alcohol). Although some specimens enumerated in Table 1 indicate that they were preserved in 95% ethanol, label data does not distinguish denatured from not denatured ethanol, and the additional collector information provides only hints of the actual preservative in most cases. Specimens preserved in 70% denatured ethanol in the field and subsequently transferred to 95% not denatured ethanol failed. Based on previous experimentation, neither acetone nor isopropyl alcohol preservation resulted in successful amplification, so these preservatives were excluded from this analysis. Similarly, specimens known to have been exposed to formalin were excluded, as all previous attempts have failed for these types of broad taxonomic, spatial, and temporal studies using Sanger sequencing approaches (RW, pers. obs.). The interactions of formalin with specimens result in denaturation of the DNA and a variety of other reactions (Tang 2006). Additionally, over time, oxida-

Table 2. Nine new mt16SrDNA Anostraca sequences: taxonomy, Genbank number, and locality information. All specimens and DNA are deposited in the collections of the Natural History Museum of Los Angeles County. Required permits are on file at USFWS and/or LACM.

Genus/species	Genbank No.	Locality
Artemiidae: *Artemia monica*	KF790567	USA, California, Mono County, Mono Lake, ~38.011°N ~119.012°W, hypersaline lake, 95% ethanol. 1 Jan 2010. Coll. M. Hauser. RW12.244.2008
Branchinectidae: *Branchinecta lindahli*	KF790568	USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.929°N, 117.22°W, vernal pool 4 in. deep, 8 ft. wide, 28 ft. long, water slightly murky, 63 µm net, 95% ethanol. 28 Dec 2011. JS pool #21, MBPC 11637. Coll. J. Snapp-Cook, C. Lieberman, A. Wall, P. Sun, R. Wetzer. RW13.042.1992
Branchinectidae: *Branchinecta lindahli*	KF790569	USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.933°N, 117.215°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 22, js_fs_37, MBPC13258. Coll. J. Snapp-Cook. RW13.047.2026
Branchinectidae: *Branchinecta lindahli*	KF790570	USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.932°N, 117.215°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 20, js_fs_38, MBPC13259. Coll. J. Snapp-Cook. RW13.048.2027
Branchinectidae: *Branchinecta lindahli*	KF790571	USA, California, San Diego County, San Diego, Carmel Mountain Preserve, 32.928°N, 117.22°W, vernal pool in dirt road, 95% ethanol. 2 Apr 2012. City ID # 26, js_fs_35, MPBC13256. Coll. J. Snapp-Cook. RW13.046.2028
Chirocephalidae: *Chirocephalus* sp.	KF790572	Mongolia, Dundgovi' aimag, near Sangiyn Dalay (Erdenedalay), 46.135°N, 105.106°E, 2 acre pond, 0-1 ppt, 23.2°C, 63 µm mesh net, 95% ethanol. 22 Aug 2002. GPS#016, Mongolia Expedition 2002, MBPC 431. Coll. R. Wetzer, S.L. Boyce, N.D. Pentcheff. RW13.034.2018
Chirocephalidae: *Eubranchipus* sp.	KF790573	USA, California, Lassen County, Poison Lake, 40.659°N, 121.197°W, temporary lake, hand, 95% ethanol. 15 May 2012. Coll. M. Hauser and D. Striley. RW12.242.2020
Streptocephalidae: *Streptocephalus woottoni*	KF790574	USA, California, San Diego County, Carlsbad, Poinsettia Lane Commuter Station Vernal Pools, large pool at southern end of complex, 33.108°N, 117.318°W, vernal pool 15 m x 30 m, 12-24 inches deep, murky water, 1 Apr 2005. MBPC 10061. Coll. J. Snapp-Cook. RW13.007.2016
Thamnocephalidae: *Phallocryptus* sp.	KF790575	Mongolia, Dundgovi' aimag, northwest of Delgerhangay (Khashaat/Delger Khanay Uul), 45.424°N, 104.481°E, large lake reduced to tiny watering hole, 11 ppt, 28°C, 63 µm mesh net, 95% ethanol. 22 Aug 2002. GPS#020, Mongolia Expedition 2002, MBPC 435. Coll. R. Wetzer, S. L. Boyce, N. D. Pentcheff. RW13.036.2009

tion of formaldehyde in formalin to formic acid produces an acidic solution resulting in the scission of DNA. The smaller the specimen, the greater the effect, and the lower the likelihood of success of long strand DNA extraction. The Tang (2006) study, commissioned by the National Academy of Sciences, provides a detailed (and discouraging) review of DNA extraction and sequencing from formalin-fixed biological samples.

Collecting recommendations

Our aim was to maximize the scientific value of specimens and their biological usefulness for future studies. First, the results of our study make a very compelling case that initial specimen fixation and preservation in the field should use 95% ethanol — *not* denatured ethanol or other alcohols. If not denatured ethanol is unavailable, we recommend fixation and preservation in 100 proof (or higher) vodka, rum, Everclear™, or similar drinking alcohol, rather than any sort of denatured alcohol. This method, although the next best choice, has been successfully used during expeditionary work by one of us (RW) since the mid-1980s. Although 100 proof spirits are only 50% ethanol by volume, the quality of the alcohol matters more than the concentration — if you cannot drink it, it's *not* good for specimens. Second, specimens should always be in a volume ratio of at least 3:1 alcohol:specimens to avoid degradation from dilution of preservative by body fluids. Third, once specimens are returned from the field, ethanol should be replaced with fresh 95% not denatured ethanol to compensate for dilution of the preservative by water extracted from specimen tissue.

In addition to the changes we suggest for the fixation and preservation, we also suggest changes to the type and number of voucher specimens being deposited after an environmental impact report is completed. We recommend accessioning specimens of all species, whether listed or not (*e.g.* whether endangered or threatened, or not). For example, simply accessioning both the listed and non-listed species will make it possible to definitively address questions about hybridization between *B. sandiegonensis* and *B. lindahli*. Furthermore, depositing all specimens collected for a survey, not just a single voucher specimen for each species, will increase sample sizes to enable population level molecular studies.

These small improvements to collecting protocols will make it possible to derive high-quality data for future biodiversity and phylogeographic research. Since the sacrifice of endangered and non-endangered crustaceans is necessary to evaluate their presence and abundance in the wild, they can become a valuable historic resource if properly curated and deposited.

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RESEARCH ARTICLE

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Diversity of the free-living marine and freshwater Copepoda (Crustacea) in Costa Rica: a review

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Abstract

The studies on marine copepods of Costa Rica started in the 1990's and focused on the largest coastal-estuarine systems in the country, particularly along the Pacific coast. Diversity is widely variable among these systems: 40 species have been recorded in the Culebra Bay influenced by upwelling, northern Pacific coast, only 12 in the Gulf of Nicoya estuarine system, and 38 in Golfo Dulce, an anoxic basin in the southern Pacific coast of the country. Freshwater environments of Costa Rica are known to harbor a moderate diversity of continental copepods (25 species), which includes 6 calanoids, 17 cyclopoids and only two harpacticoids. Of the +100 freshwater species recorded in Central America, six are known only from Costa Rica, and one appears to be endemic to this country. The freshwater copepod fauna of Costa Rica is clearly the best known in Central America. Overall, six of the 10 orders of Copepoda are reported from Costa Rica. A previous summary by 2001 of the free-living copepod diversity in the country included 80 marine species (67 pelagic, 13 benthic). By 2009, the number of marine species increased to 209: 164 from the Pacific (49% of the copepod fauna from the Eastern Tropical Pacific) and 45 from the Caribbean coast (8% of species known from the Caribbean Basin). Both the Caribbean and Pacific species lists are growing. Additional collections of copepods at Cocos Island, an oceanic island 530 km away of the Pacific coast, have revealed many new records, including five new marine species from Costa Rica. Currently, the known diversity of marine copepods of Costa Rica is still in development and represents up to 52.6% of the total marine microcrustaceans recorded in the country. Future sampling and taxonomic efforts in the marine habitats should emphasize oceanic environments including deep waters but also littoral communities. Several Costa Rican records of freshwater copepods are likely to represent undescribed species. Also, the biogeographic relevance of the inland copepod fauna of Costa Rica requires more detailed surveys.

Keywords

New records, biodiversity, Pacific, Caribbean, microcrustaceans, biogeography

Introduction

Copepods are a highly diverse group of crustaceans; over 13 000 species of this subclass have been described (Boxshall and Halsey 2004, Boxshall and Defaye 2008) and many more are added each year. Copepods are also one of the most abundant metazoans in the world. Up to 60–80% of the marine zooplankton biomass in neritic and oceanic environments is represented by copepods (Casanova et al. 1982). This abundance is related to the success of the planktonic forms in colonizing the oceanic water column (Boxshall and Halsey 2004). Huys and Boxshall (1991) summarized the economic and biological importance of copepods.

The free-living forms inhabit a wide variety of aquatic environments including also those with extreme conditions of salinity and temperature. They are common members of the biotas of subterranean waters (Pipan 2005), anchialine systems (Suárez-Morales and Iliffe 2005), hypersaline waters (Brucet et al. 2009), and bromeliads, among others (Reid 1986).

Although the importance of the free-living copepods is clear, information on their diversity and distribution is scarce and scattered in many regions. In the case of marine forms, Mauchline (1998) listed 13 Large Marine Ecosystems (LME) that have some data regarding their copepod fauna, but information on most of the remaining 75% of LMEs remains largely incomplete. Similarly, the freshwater environments harbor a diverse copepod fauna; in general, the knowledge of the group in the Americas is asymmetrical, Central America being the least studied subregion in the continent. As part of the Neotropical region with a high continental copepod diversity (Boxshall and Defaye 2008), studies of the group in Costa Rica have been intermittent (Collado et al. 1984a, b, Suárez-Morales and Reid, unpubl. data), but new efforts are revealing interesting records (Suárez-Morales et al. 2011).

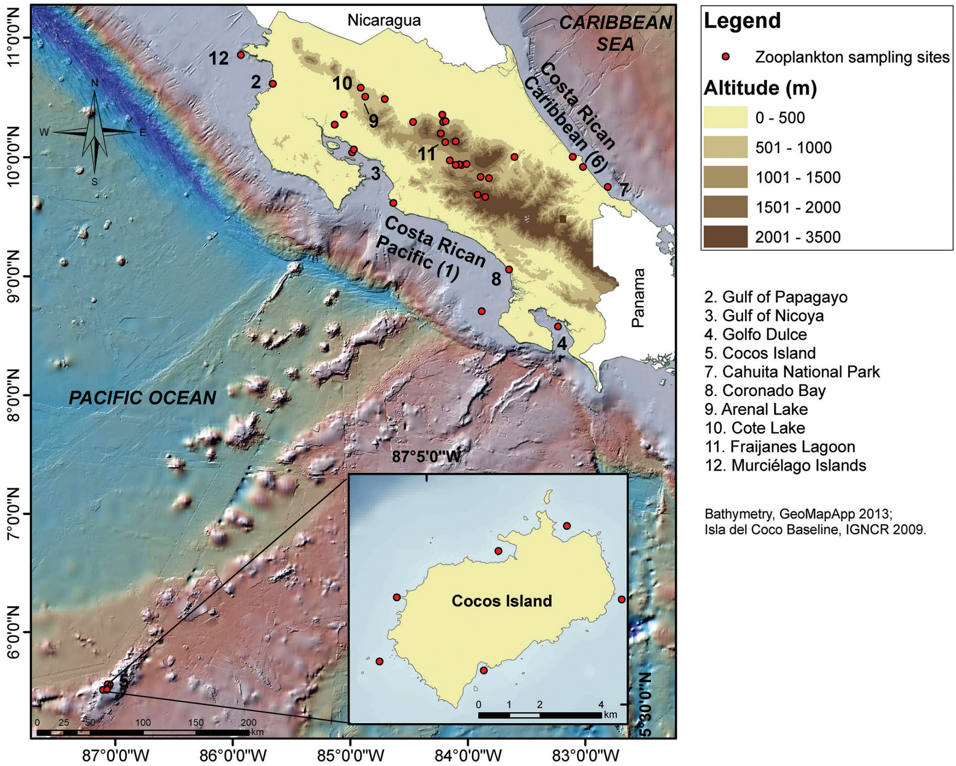
A periodical revision of the progress of the knowledge of the copepod fauna in Costa Rica is a key tool to evaluate their potential diversity in marine and freshwater environments. It is also useful to detect invasive species in different aquatic habitats, which is a worldwide phenomenon representing a serious threat to the aquatic biodiversity (Molnar et al. 2008). The present paper summarizes the information about copepods that has been recorded until now in Costa Rica, a country with two coastlines and with a high habitat diversity.

Methods

Surveyed aquatic systems in Costa Rica

The main coastal and oceanic environments and also the lakes and freshwater sites in which copepods have been surveyed in Costa Rica are presented in Figure 1. These areas and systems were selected because of their physiographic or ecological features: the northern Pacific coast is influenced by the Gulf of Papagayo jet-driven upwelling system (Mc Creary et al. 1989, Chelton et al. 2000). The Gulf of Nicoya, located in the central Pacific Costa Rican coast is one of the largest and well-studied tropical estuaries, with a surface area of 1530 km^2 (Vargas 1995), and Golfo Dulce to the south is an anoxic fjord-like embayment (Svendsen et al. 2006). The Cocos Island is the only emergent point of an oceanic submarine ridge (Protti et al. 2012), situated 496 km off Cabo Blanco, Pacific coast, with a important marine diversity (Cortés 2012), and since 1997 a UNESCO World Heritage Site (Morales-Ramírez 2008). The Caribbean coast is represented mainly by river deltas dominated by waves and barrier beaches to the north, and sandy beaches alternating with a few formations of coral reef fossils to the south (Denyer and Cárdenas 2000). The most studied freshwater bodies are Lake Arenal, studied since the 1980's, and smaller lakes like Cote and Fraijanes (Collado et al. 1984a, b, Umaña and Collado 1990).

We reviewed literature records of free-living marine, brackish water, and freshwater copepods found in Costa Rica. Up to thirty seven sites have been sampled for copepods in the country, including marine and freshwater environments (Fig. 1). Marine pelagic copepods were sampled using different gears including WP-2 standard zooplankton nets with mesh sizes of 100, 150, 200, and 500 µm. Vertical tows were performed at depths less than 100 m in Golfo Dulce and Cocos Island (Morales-Ramírez and Nowaczyk 2006, Quesada-Alpízar and Morales Ramírez 2006, Morales-Ramírez 2008). The same kind of tows were used to sample copepods in the Gulf of Papagayo (Bednarski and Morales-Ramírez 2004, Rodríguez-Saénz and Morales-Ramírez 2012), Gulf of Nicoya and Coronado Bay (Morales-Ramírez 1996). In the Caribbean Sea, organisms were collected mostly with horizontal surface tows (Morales-Ramírez and Murillo 1996, Carrillo 2012). For marine benthic copepods, sampling techniques were described by Mielke (1992, 1994 a,b,c). This community was also sampled by collecting sediment samples with cores from beaches (Mielke 1992, 1994a, b, c, 1995), but also by a van Veen grab sampler (Sibaja-Cordero 2012). Freshwater copepods were obtained by different devices but mainly by nets used in different habitats including littoral and limnetic areas of the water bodies (see Collado et al. 1984a, b, Suárez-Morales and Reid, unpubl. data).



Bathymetry, GeoMapApp 2013;
Isla del Coco Baseline, IGNCR 2009.

Figure 1. Maps with the sampling sites for marine and freshwater copepods in Costa Rica.

Results and discussion

Historical overview

Marine pelagic copepods

The copepod species found in marine systems of Costa Rica are listed in Table 1. The zooplankton of the Pacific coast of Costa Rica has been surveyed more intensely and during a longer period of time than in the Caribbean side. The three sectors (northern, central and southern) of the Pacific coast include interesting systems in which the copepod fauna has been studied. In Culebra Bay, an embayment associated to the Gulf of Papagayo (north Pacific coast), Bednarski (2001) recorded 39 species. Suárez-Morales and Morales-Ramírez (2001) reported the calanoid *Acartia negligens* Dana, 1849 near Murciélago Islands at the northern Pacific coast of Costa Rica and expanded its known regional range. In the same region, a new species (*Cymbasoma concepcionae* Suárez-Morales and Morales-Ramírez 2003) of the order Monstrilloida was described; it was the first record of monstrilloids in Costa Rica. Also in the northern Pacific sector, an oceanic upwelling system known as the Costa Rica Dome has been the subject of many zooplankton studies because of its importance as a fisheries region (Fiedler 2002).

Table 1. List of Copepoda found in marine environments of Costa Rica. Records from published works of the Costa Rican marine copepod fauna. Localities in Costa Rica: 1 – Costa Rican Pacific, 2 – Gulf of Papagayo, 3 – Gulf of Nicoya, 4 – Golfo Dulce, 5 – Cocos Island, 6 – Costa Rican Caribbean, 7 – Cahuita National Park. References: 1. Morales-Ramírez and Suárez-Morales (2009), 2. Carrillo (2012), 3. Sibaja-Cordero (2012), 4. Morales-Ramírez et al. (unpubl. data), 5. Morales-Ramírez and Vargas (1995), 6. Suárez-Morales et al. (2013), 7. Suárez-Morales and Morales-Ramírez (2009), 8. Morales-Ramírez (2001).

Family	Species	Locality	References
Gymnoplea			
Order Calanoida			
Acartiidae	*Acartia clausi* Giesbrecht, 1889	1, 3, 4, 5	1, 4
	Acartia danae Giesbrect, 1889	1, 3, 4, 5	1, 4
	Acartia lilljeborgii Giesbrecht, 1889	1, 2, 3, 4, 5	1, 4
	Acartia negligens Dana, 1849	2	1
Aetideidae	*Aetideopsis rostrata* G.O. Sars, 1905	1	1
	Aetideus armatus Boeck, 1872	1	1
	Aetideus giesbrechti (Cleve, 1904)	5	4
	Chiridius Giesbrecht, 1892	1	1
	Euchirella brevis G.O. Sars, 1905	1	1
	Euchirella rostrata Claus, 1866.	7	1
	Euaetideus giesbrechti Sars, 1925	1	1
	Gaetanus brevispinus Sars, 1903	1	1
	Gaetanus miles Giesbrecht, 1888	1	1
	Gaetanus minor Farran, 1905	1	1
	Gaidius tenuispinus Sars, 1900	1	1
	Valdiviella brevicornis Steuer, 1904	1	1
Arietellidae	*Arietellus* sp. Giesbrecht, 1892	1	1
Augaptilidae	*Augaptilus longicaudatus* Giesbrecht, 1889	1	1
	Augaptilus megalurus Giesbrecht, 1889	7	2
	Haloptilus acutifrons Giesbrecht, 1892	1	1
	Haloptilus longicornis Claus, 1863	1, 6	1
	Haloptilus mucronatus Claus, 1863	1	1
	Haloptilus ornatus Giesbrecht, 1892	1	1
	Haloptilus oxycephalus Giesbrecht, 1889	1	1
Calanidae	*Neocalanus cristatus* (Krøyer, 1848)	1	1
	Calanus pacificus Brodsky, 1948	5	4
	Mesocalanus tenuicornis (Dana, 1849)	1	1
	Canthocalanus pauper (Giesbrecht, 1888)	1, 2, 3, 4, 5	1, 4
	Cosmocalanus darwini (Lubbock, 1860)	1, 5	1, 4
	Nannocalanus minor Claus, 1863	1, 3, 4, 5	1, 4
	Neocalanus gracilis Dana, 1849	1, 2, 5	1, 4
	Neocalanus robustior (Giesbrecht, 1888)	1, 5	1, 4
	Undinula vulgaris Dana, 1842	1, 2, 3, 4, 5, 6, 7	1, 2, 4
Calocalanidae	*Calocalanus pavo* Dana, 1849	1, 5, 7	1, 2, 4
	Calocalanus pavoninus Farran, 1926	1	1
	Calocalanus styliremis Giesbrecht, 1888	1, 3, 4	1
	Calocalanus contractus Farran, 1926	1	1
	Ishnocalanus plumulosus Claus, 1863	1	1
Candaciidae	*Candacia catula* Giesbrecht, 1881	1, 2, 3, 5	1, 4

Family	Species	Locality	References
	Candacia truncata Dana, 1846	1, 5	1, 4
	Candacia pachydactyla Dana, 1849	1, 5	1, 4
	Candacia varicans Giesbrecht, 1892	1	1
Centropagidae	*Centropages abdominalis* (Sato, 1913)	5	4
	Centropages bradyi (Wheeler, 1900)	5	4
	Centropages calaninus (Dana, 1849)	1, 5	1, 4
	Centropages furcatus (Dana, 1849)	1, 5	1, 4
	Centropages gracilis (Dana, 1849)	5	4
	Centropages longicornis Mori, 1932	1, 5	1, 4
Clausocalanidae	*Clausocalanus arcuicornis* (Dana, 1849)	1, 5	1, 4
	Clausocalanus furcatus Brady, 1883	1, 5	1, 2, 4
	Clausocalanus pergens Farran, 1926	1, 3, 4	1
Eucalanidae	*Eucalanus attenuatus* Dana, 1849	1, 5	1, 4
	Eucalanus bungii Giesbrecht, 1892	1	1
	Eucalanus crassus (Giesbrecht, 1888)	7	2
	Eucalanus elongatus Dana, 1849	1	1
	Eucalanus inermis Griesbrecht, 1892	1	1
	Eucalanus monachus Giesbrecht, 1888	6	1
	Eucalanus mucronatus Giesbrecht, 1891	1	1
	Eucalanus pileatus Giesbrecht, 1888	1	1
	Eucalanus sewelli Fleminger, 1973	1, 5	1, 4
	Eucalanus subcrassus Giesbrecht, 1888	5, 6	1, 2, 4
	Eucalanus subtenuis Giesbrecht, 1888.	1, 5	1, 4
	Rhincalanus cornutus Dana, 1849.	5, 6	1, 4
	Rhincalanus nasutus Giesbrecht, 1888	1, 5	1, 4
Euchaetidae	*Euchaeta acuta* Giesbrecht, 1892	1	1
	Euchaeta plana Philippi, 1843	5	4
	Euchaeta barbata Brady, 1883	1	1
	Euchaeta indica Wolfenden, 1905	1, 5	1, 4
	Euchaeta longicornis Giesbrecht, 1888	1, 5	1, 4
	Euchaeta marina (Prestandrea, 1833)	1, 5	1, 4
	Euchaeta media Giesbrecht, 1888	1	1
	Euchaeta rimana (Bradford, 1974)	5	4
	Euchaeta tenuis Esterly, 1906	1	1
	Euchaeta wolfendeni Scott, 1909	1	1
	Paraeuchaeta hansenii (With, 1915)	1	1
	Paraeuchaeta norvegica (Boeck, 1872)	1	1
	Paraeuchaeta tonsa (Giesbrecht, 1895)	1	1
Heterorhabdidae	*Heterorhabdus papilliger* Claus, 1863	1	1
	Scaphocalanus sp. G.O. Sars, 1900	1	1
Lucicutiidae	*Lucicutia bicornuta* Wolfenden, 1911	1	1
	Lucicutia flavicornis Claus, 1963	1, 5, 6	1, 4
	Lucicutia gemina Farran, 1926	1	1
	Lucicutia grandis Giesbrecht, 1895	1	1
	Lucicutia ovalis Giesbrecht, 1889	1	1
Mecynoceridae	*Mecynocera clausi* Thompson, 1888	1, 5	1, 4
Metridinidae	*Metridia* sp. Boeck, 1864	1	1
	Pleuromamma abdominalis f. *edentata* Steuer, 1931	1	1

Family	**Species**	**Locality**	**References**
	Pleuromamma abdominalis f. *abyssalis* Steuer, 1931	1	1
	Pleuromamma abdominalis abdominalis Lubbock, 1856	1	1
	Pleuromamma gracilis Claus, 1863	1, 5	1, 4
	Pleuromamma piseki Farran, 1929	1	1
	Pleuromamma quadrungulata Dahl, 1893	1	1
	Pleuromamma robusta Dahl, 1893	1	1
	Pleuromamma scutullata Brodsky, 1950	1	1
	Pleuromamma xiphias (Giesbrecht, 1889)	1	1
Paracalanidae	*Acrocalanus gibber* Giesbrecht, 1888	1, 3, 4, 5	1, 4
	Acrocalanus gracilis Giesbrecht, 1888	1, 5	1, 4
	Acrocalanus longicornis Giesbrecht, 1888	1	1, 2
	Paracalanus aculeatus Giesbrecht 1888	1, 5	1, 2
	Parvocalanus crassirostris Dahl, 1894	1	1
	Paracalanus parvus Claus, 1863	1	1
Phaennidae	*Cephalophanes* sp. Sars, 1907	1	1
	Cornucalanus sp. Wolfenden, 1905	1	1
	Phaenna spinifera Claus, 1863	1, 5	1, 4
Pontellidae	*Calanopia americana* F. Dahl, 1894	1, 5, 6	1, 4
	Labidocera acuta Dana, 1849	1, 5	1, 4
	Labidocera aestiva Wheeler, 1901	1, 5, 7	1, 2, 4
	Labidocera dentruncata (Dana, 1849)	5	4
	Labidocera lubboki Giesbrecht, 1892	1	1
	Labidocera scotti Giesbrecht, 1897	7	1, 2
	Pontella agassizii Giesbrecht, 1895	1	1
	Pontella mimocerami Fleminger 1957	6	1
	Pontellina plumata Dana, 1849	1, 5	1, 4
	Pontellopsis villosa Brady, 1883	1	1
	Pontellopsis yumadae (Mori 1937)	5	4
Pseudodiaptomidae	*Pseudodiaptomus acutus* Dahl, 1894	6	1
	Pseudodiaptomus cristobalensis Marsh, 1919	1	1
	Pseudodiaptomus marshi Wright, 1936	6	1
	Pseudodiaptomus panamensis Walter, 1989	3	8
	Pseudodiaptomus wrigthi Johnson, 1964	1	1
Scolecithricidae	*Amallothrix gracilis* Sars, 1925	1	1
	Lophothrix sp. Giesbrecht, 1895	1	1
	Scolecithricella dentata (Giesbrecht, 1892)	1	1
	Scolecithricella marginata (Giesbrecht, 1888)	1, 3, 4	1
	Scolecithricella tenuiserrata (Giesbrecht, 1892)	1	1
	Scolecithricella vittata (Giesbrecht, 1892)	1	1
	Scolecithricella bradyi (Giesbrecht, 1888)	1, 5	1
	Scolecithrix danae Lubbock, 1856	1, 5, 6	1, 4
	Scottocalanus sp. Sars, 1905	1	1
Temoridae	*Eurytemora* Giesbrecht, 1881	1	1
	Temora discaudata Giesbrecht, 1889	1, 5	1, 4
	Temoropia mayumbaensis Scott, 1894	1, 3, 4, 5	1, 4
	Temora stylifera (Dana, 1849)	5, 7	2, 4
	Temora turbinata Dana, 1849	1	1, 2

Family	**Species**	**Locality**	**References**
Super Order PODOPLEA			
Order CYCLOPOIDA			
Corycaeidae	*Corycaeus bremhi* Dana, 1849	1	1
	Corycaeus catus Dana, 1845	5	4
	Corycaeus clausi Dahl F., 1894	7	2
	Corycaeus crassiusculus (Dana, 1848)	5	4
	Corycaeus (*Agetus*) *flaccus* Giesbrecht, 1891	1, 3, 4	1
	Corycaeus furcifer (Claus, 1863)	5	4
	Corycaeus latus (Dana, 1848)	5	4
	Corycaeus limbatus Brady, 1883	7	2
	Corycaeus (*Corycaeus*) *speciosus* Dana, 1849	1, 5, 7	1, 2, 4
	Corycaeus robustus (Giesbrecht, 1891)	5	4
	Corycaeus (*Onychocorycaeus*) *ovalis* Claus, 1863	1, 5	1, 4
	Farranula carinata Giesbrecht, 1891	5	4
	Farranula gibbula Giesbrecht, 1981	1, 5	1, 4
	Farranula gracilis Dana, 1849	6	1
Oithonidae	*Oithona hebes* Giesbrecht, 1891	6	1
	Oithona nana Giesbrecht, 1893	6	2
	Oithona plumifera Bair, 1843	1, 5, 6	1, 4
	Oithona setigera Claus, 1863	1, 6	1, 2
	Oithona similis Claus, 1863	1, 5, 6	1, 4
	Oithona spinirostris Claus, 1863	1	1
Oncaeidae	*Conaea gracilis* Dana, 1853	1, 6	1
	Lubbockia aculeata Giesbrecht, 1892	1	1
	Oncaea conifera Giesbrecht, 1891	1, 5	1, 4
	Oncaea mediterranea Claus, 1883	1, 5	1, 4
	Oncaea ornata Giesbrecht, 1891	1	1
	Oncaea venusta Phillippi, 1843	1, 5, 7	1, 4
	Pachos punctatum (Claus, 1863)	5	4
Clausidiidae	*Hemicyclops thalassius* Vervboort & Ramírez, 1966	3	5
Sapphirinidae	*Copilia longistylis* (Mori, 1932)	5	4
	Copilia mirabilis Dana, 1852	5, 7	2, 4
	Copilia quadrata (Dana, 1852)	5	4
	Copilia vitrea Haeckel, 1864	1, 5	1, 4
	Sapphirina angusta (Dana, 1849)	5	4
	Sapphirina darwinii (Haeckel, 1864)	5	4
	Sapphirina gastrica (Giesbrecht, 1891)	5	4
	Sapphirina metallina (Dana, 1849)	5	4
	Sapphirina nigromaculata Claus, 1863	1, 5	1, 2
	Sapphirina opalina Dana, 1849	1, 5	1, 4
	Sapphirina ovatolanceolata Dana, 1852	1, 5	1, 4
	Sapphirina scarlata Giesbrecht, 1891	1, 5	1, 4
	Vettoria sp. Wilson C.B., 1924		
Order MONSTRILLOIDA			
Monstrillidae	*Cymbasoma alvaroi* Suárez-Morales & Carrillo, 2013	7	6
	Cymbasoma cocoense Suárez-Morales & Morales-Ramírez, 2009	5	7
	Cymbasoma concepcionae Suárez-Morales & Morales-Ramírez, 2003	1	1

Family	Species	Locality	References
	Monstrilla grandis Giesbrecht, 1891	7	6
	Monstrillopsis cahuitae Suárez-Morales, Carrillo & Morales-Ramírez, 2013	7	6
	Monstrillopsis chathamensis Suárez-Morales & Morales-Ramírez, 2009	5	7
Order MORMONILLOIDA			
Mormonillidae	*Mormonilla minor* Giesbrecht, 1891	1	1
	Mormonilla phasma Giesbrecht, 1891	1	1
Order HARPACTICOIDA			
Aegisthidae	*Aegistus aculeatus* Giesbrecht, 1891	6	1
Canuellidae	*Microcanuella bisetosa* Mielke, 1994	1	1
Cletodidae	*Cletodes* sp. Brady, 1872	5	3
Clytemnestridae	*Clytemnestra rostrata* Brady, 1883	1, 5	1, 2, 4
	Clytemnestra scutellata Dana, 1847	1, 5	1, 2, 4
Ectinosomatidae	*Halectinosoma* sp. Vervoot, 1962	5	3
	Microsetella cf. *norvegica* (Boeck, 1865)	5	3
	Microsetella rosea Dana, 1848	1, 5	1, 4
Diosaccidae	*Balucopsylla triarticulata* Wells & Rao, 1987	1	1
	Schizopera nicoyana Mielke, 1995	3	1
	Schizopera osana Mielke, 1995	1	1
	Schizopera sp. *A* Mielke, 1995	1	1
	Schizopera sp. *B* Mielke, 1995	1	1
Harpacticidae	*Zausodes septimus* Lang, 1965	1	1
Laophontidae	*Afrolaophonte schmidti* Mielke, 1997	1	1
	Klienychocamptoides itoi Mileke, 1981	1	1
	Laophontella horrida dentata Por, 1964	1	1
	Mexicolaophonte arganoi Cottarelli, 1977	1	1
Leptastacidae	Leptastacidae undet.	5	3
Longipediidae	*Longipedia helgolandica* Klie, 1949	6	1
Miraciidae	*Amonardia* sp. Lang, 1944	5	3
	Amphiascopsis cf. *cinctus* (Claus, 1866)	5	3
	Macrosetella gracilis Dana, 1852	1, 5	1, 2, 4
	Robertgurneya sp. Lang, 1944	5	3
	Typhlamphiascus sp. Lang, 1944	5	3
Orthopsyllidae	*Orthopsyllus linearis curvaspina* Claus, 1886	5	3
Paramesochridae	Paramesochridae indet.	5	3
Peltidiidae	*Peltidium nichollsi* Geddes, 1968	6	2
Phyllopodidae	*Phyllopodopsyllus ancylus* Mielke, 1992	1	1
	Phyllopodopsyllus carinatus Mielke, 1992	1	1
	Phyllopodopsyllus gertrudi costaricensis Kunz, 1984	1	1
	Phyllopodopsyllus setouchiensis Kitazima, 1981	1	1
Euterpinidae	*Euterpina acutifrons* (Dana, 1847)	1, 5, 6	1
Thalestridae	*Amenophia* sp. Boeck, 1865	5	2
Tetragonicipitidae	*Oniscopsis robinsoni* Chappuis & Delamare, 1956	6	1

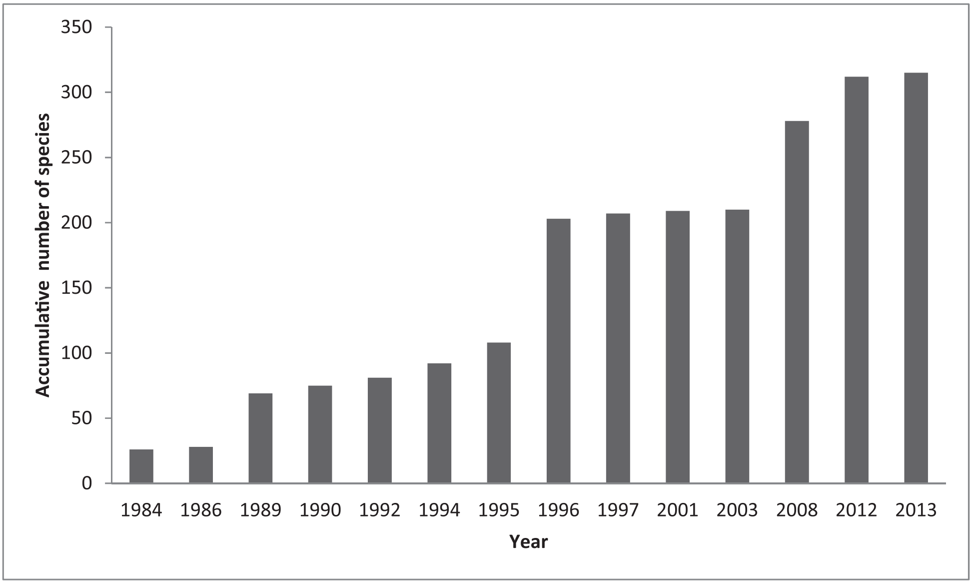


Figure 2. Accumulative number of species of marine and freshwater copepods recorded per year in Costa Rica. Data from the revision of the published literature.

A total of 41 copepod species have been recorded in surface waters of this highly productive area (Suárez-Morales and Gasca 1989, Fernández-Alamo and Vicencio 1996).

In the Gulf of Nicoya, an estuarine system on the central Pacific coast of Costa Rica, Morales-Ramírez and Vargas (1995) reported 12 copepod species dwelling at the inner sector of the gulf. They determined *A. lilljeborgii* and members of the family Pseudodiaptomidae as the most abundant taxa. Further studies in the Gulf of Nicoya raised the total number of species to 32 (Morales-Ramírez 1996).

Zooplankton studies in the southern Pacific area of Costa Rica have focused on two locations. The first one is Coronado Bay, which is part of the Térraba-Sierpe mangrove system. Its copepod fauna comprised 13 species of a few families, mainly Clausocalanidae, Calanidae and Paracalanidae (Morales-Ramírez 1996). The second location is Golfo Dulce, where Morales-Ramírez (1996) recorded 38 species. The genera *Corycaeus*, *Clausocalanus*, *Oncaea* and *Oithona* were the most common in this area. This gulf harbors 21.5% of the marine diversity recorded from the Costa Rican Pacific coast (Morales-Ramírez 2011).

In the Caribbean coast of Costa Rica, studies on copepods are scarcer and more recent. In the Cahuita National Park, Morales-Ramírez (2001) collected 22 species, with *A. lilljeborgii* and species of the family Pontellidae as the dominant forms. At that time, 18 of those species were reported as new records for the Caribbean coast of Costa Rican. Further studies revealed 13 additional records (Carrillo 2012) and two new species of monstrilloid copepods: *Monstrillopsis cahuitae* Suárez-Morales, Carrillo & Morales-Ramírez, 2013 and *C. alvaroi* Suárez-Morales & Carrillo, 2013 (Suárez-Morales et al. 2013).

The zooplankton sampling efforts in Costa Rica have been carried out since the the 1980´s. Figure 2 shows the accumulative number of species progressively found by national investigators. There seems to be major contributions to a sustained increase since 1984, nevertheless, the period between 1996 and 2003 added few or none new records; subsequently, with the beginning of studies around Cocos Island National Park increased the number of records. The first studies that included some analysis of copepods as a group (not on a species level) were from the coastal area around the Cahuita National Park, a Caribbean reef system (Morales 1987), and Caño Island at the Pacific coast (Guzmán and Obando 1988). Considering these early surveys, it is concluded that the current knowledge of the copepod fauna comprises more than two decades (Fig. 2). In 2009, the list of species recorded in Costa Rica included 209 species of marine copepods: 185 were planktonic and 24 benthic, 165 in the Pacific coast and 44 in the Caribbean waters. These numbers excluded species from Cocos Island (Morales-Ramírez and Suárez-Morales 2009).

Periodical sampling of the zooplankton of Cocos Island started in 2007, with a 6-station sampling plan around the island and in coral reefs patches. These samples first yielded 68 species of copepods (Morales-Ramírez 2008), a list that now includes 95 species (Table 1), and 14 new records of planktonic copepods, eight benthic forms (Morales-Ramírez et al. unpubl. data), and two new monstrilloids species: *C. cocoense* Suárez-Morales & Morales-Ramírez, 2009 and *M. chathamensis* Suárez-Morales & Morales-Ramírez, 2009; the last case represented the first record of the genus *Monstrillopsis* for Costa Rican waters (Suárez-Morales and Morales-Ramírez 2009). The species composition of the copepod fauna from Cocos Island and adjacent oceanic waters has been summarized by Morales-Ramírez (2008). The two most diverse families are Corycaeidae and Sapphirinidae (unpubl. data).

Marine benthic copepods

The number of free-living copepod taxa that have been described from material obtained in Costa Rica is summarized in Table 2. Taxonomic surveys of the benthic copepod fauna are scarce. Mielke (1992) analyzed beach sediments from locations along both coasts of Costa Rica: along the Caribbean coast, samples were obtained at three locations (Portete, Cahuita and Manzanillo beaches), whereas at the Pacific coast, samples were obtained from 13 sites. Six copepod taxa belonging to the family Tetragonicipitidae were found, including two new species (*Phyllopodopsyllus ancylus* Mielke, 1992 and *P. carinatus* Mielke, 1992) and two new subspecies (*Laophontella horrida dentata* Mielke, 1992 and *P. gertrudi costaricensis* Mielke, 1992). The monotypic genus *Microcanuella* and its species *M. bisetosa* Mielke, 1994 were described from sandy beaches of the Gulf of Nicoya. Mielke (1995) also found four species of *Schizopera* Sars G.O., 1905 and described *Schizopera osana* Mielke, 1995.

At Cocos Island, Sibaja-Cordero (2012) analyzed subtidal sediment samples and identified seven families, nine genera and three species of harpacticoid copepods. The family Miraciidae included four genera, one species and an undetermined taxon. This material is expected to reveal many additional species records of the Harpacticoida.

Table 2. New species described from type material collected in Costa Rica. References. 1. Mielke (1994), 2. Mielke (1992), 3. Mielke (1994c), 4. Mielke (1995), 5. Suárez-Morales and Morales-Ramírez (2003), 6. Suárez-Morales and Morales-Ramírez (2009), 7. Suárez-Morales et al. (2013), 8. Suárez-Morales and Gasca (2012).

Order	Species	Location	Environment	References
Harpacticoida	*Karllangia obscura* Mielke, 1994	Manzanillo, Caribbean coast	Mud flat	1
	Karllangia pulchra Mielke, 1994	Manzanillo, Caribbean coast	Mud flat	1
	Phyllopodopsyllus carinatus Mielke, 1992	Manzanillo, Caribbean coast	Beach slope	2
	Phyllopodopsyllus gertrudi costaricensis Mielke, 1992	Manzanillo, Caribbean coast	Beach slope and mangrove	2
	Phyllopodopsyllus ancylus Mielke, 1992	Punta Morales, Pacific coast	Mud flat	2
	Orthopsyllus linearis curvaspina (Claus, 1886)	Pacific coast	Sandy beach	3
	Microcanuella bisetosa Mielke, 1994	Gulf of Nicoya, Pacific coast	Sandy beach	3
	Schizopera nicoyana Mielke, 1995	Gulf of Nicoya, Pacific coast	Sandy beach	4
	Schizopera osana Mielke, 1995	Gulf of Nicoya, Pacific coast	Sandy beach	4
	Laophontella horrida dentata (Por, 1964)	Pacific coast	Sandy beach	2
Monstrilloida	*Cymbasoma concepcionae* Suárez-Morales & Morales-Ramírez, 2003	Bahía Culebra, Pacific coast	pelagic	5
	Cymbasoma cocoense Suárez-Morales & Morales-Ramírez, 2009	Cocos Island, Pacific Ocean	pelagic	6
	Monstrillopsis chathamensis Suárez-Morales & Morales-Ramírez, 2009	Cocos Island, Pacific coast	pelagic	6
	Monstrillopsis cahuitae Suárez-Morales, Carrillo & Morales-Ramírez, 2013	Cahuita National Park, Caribbean coast	pelagic	7
	Cymbasoma alvaroi Suárez-Morales, Carrillo & Morales-Ramírez, 2013	Cahuita National Park, Caribbean coast	pelagic	7
Siphonostomatoida	*Lepeophtheirus alvaroi* Suárez-Morales & Gasca, 2012	Cocos Island, Pacific Ocean	water column	8

Freshwater copepods

The freshwater zooplankton of Costa Rica has been studied since the 1980's, but these surveys have been intermittent (Collado et al. 1984a, b, Dussart and Fernando 1986, Reid 1990a, Umaña and Collado 1990, Haberyan et al. 1995). Since then, a total of 25 species of copepods have been recorded (Table 3). Cyclopid copepods are the most diverse and abundant group in inland waters of Costa Rica as found in other Neotropical areas (Suárez-Morales et al. 2000, Silva 2008). Diaptomid copepods, the most representative group of the order Calanoida in freshwater environments, show a low diver-

Table 3. Species of freshwater copepods recorded in Costa Rica (* new range extension includes Costa Rica, ** only known from Costa Rica). References: 1. Collado et al. (1984a), 2. Collado et al. (1984b), 3. Dussat and Fernando (1986), 4. Reid (1990a), 5. Reid (1990b), 6. Gavlas (2012), 7. Suárez-Morales et al. (2013).

Family	Species	References
Gymnoplea		
Order Calanoida		
Diaptomidae	*Arctodiaptomus dorsalis* (Marsh, 1907)	2
	Diaptomus sp. Westwood, 1836	1
	Prionodiaptomus colombiensis (Thiébaud, 1912)*	6
Super Order PODOPLEA		
Order CYCLOPOIDA		
Cyclopidae	*Ectocyclops pharelatus* (Koch, 1838)	1
	Eucyclops agilis (Koch, 1838)	1
	Eucyclops bondi Kiefer, 1934	2
	Eucyclops leptacanthus Kiefer, 1956	2
	Eucyclops serrulatus (Fischer, 1851)	1
	Halicyclops exiguus Kiefer, 1934	1
	Macroyclops albidus (Jurine, 1820)	1
	Mesocyclops brasilianus Kiefer, 1933	1
	Mesocyclops leuckarti (Claus, 1857)	1
	Mesocyclops thermocyclopoides Harada, 1931	2,7
	Microcyclops ceibaensis (Marsh, 1919)	2
	Microcyclops dubitabilis Kiefer, 1934	2
	Microcyclops varicans (G.O Sars, 1863)	1
	Paracyclops fimbriatus (Fischer, 1853)	2
	Thermocyclops crassus (Fischer, 1853)	2
	Thermocyclops decipiens (Kiefer, 1929)	2
	Thermocyclops inversus (Kiefer, 1936)	2
	Thermocyclops tenuis (Marsh, 1910)	2
	Tropocyclops prasinus (Fishcer, 1860)	2
	Tropocyclops pseudoparvus Dussart & Fernando, 1986**	3
Order HARPACTICOIDA		
Canthocamptidae	*Attheyella fuhrmanni* (Thiébaud, 1912)	4
	Canthocamptus striblingi (Reid, 1990) **	5

sity in Costa Rica, a feature which is also shared with Central America (Suárez-Morales 2003, Suárez-Morales et al. 2005). Their diversity could be underestimated in Costa Rica; records of presumedly cosmopolitan species like *Eucyclops agilis*, *E. serrulatus* could include undescribed species (Alekseev et al. 2006, Gutiérrez-Aguirre et al. 2013). The Costa Rican *E. bondi* probably represents an undescribed species (Mercado-Salas and Suárez-Morales 2014).

General diversity

Studies focusing on the taxonomic composition of the free-living copepod fauna have been carried out in selected locations involving coastal, oceanic and large continental aquatic systems (i.e., lakes as: Arenal, Cote, Bonilla, Fraijanes, and Cerro Chato). Currently, there are 281 species of copepods recorded in Costa Rica, representing 6 orders and 50 families. The order with the highest number of species is Calanoida (147 spp.), followed by Cyclopoida (73) and Harpacticoida (36). There are no records of species of the orders Gelyelloida, Platycopioida and Misophrioida.

According to our results, copepods are the most studied marine group of microcrustaceans in Costa Rica, representing almost 44% of the 473 marine species reported in the country until 2009 (Wehrtmann and Cortés 2009); this figure increases to 52.6% when studies around Cocos Island and Caribbean Sea as well new records from other areas are considered.

Remarks

Marine copepods

Being situated in a fully tropical area with the influence of both the Atlantic and Pacific oceans, the marine copepod fauna of Costa Rica is expected to be highly diverse. The analysis of the diversity is also an important tool to reveal changes and patterns of the pelagic communities surveyed. In these coastal systems, particularly along the Pacific coast, the diversity of copepods has detectable variations as a response to local oceanographic conditions. Also, typical upwelling species have been recorded in Culebra Bay, Gulf of Papagayo, with major seasonal changes in dominance as a response to upwelling conditions (Bednarski and Morales-Ramírez 2004). The Gulf of Nicoya is known to show an assemblage of typical estuarine species, where small calanoids dominate year-round (Brugnoli et al. 2004). The Golfo Dulce represents a mixed environment in which oceanic and coastal copepod species coexist, a condition that is intensified during El Niño events (Quesada-Alpízar and Morales-Ramírez 2006). These and other Costa Rican systems should be surveyed during different seasons and hydrographic conditions in order to develop a complete overview of their copepod diversity. Also, increased efforts should be carried out to explore the diversity of benthic copepods, mainly of harpacticoids, a group whose diversity in marine and freshwater environments is still poorly known in Costa Rica.

Freshwater copepods

Currently, 25% of Central American records of freshwater species are from Costa Rica. Together with that of the other areas of Central America (CA), its inland copepod fauna represents an interesting assemblage, because CA is a recent biogeographic passage of copepods between North and South America (Suárez-Morales 2003, Suárez-Morales et al. 2005). Also, there are records of introduced species of copepods in Costa Rica (Collado et al. 1984a, b, Suárez-Morales et al. 2011); their advancement in the country should be followed based on an expanded sampling program. Additional efforts are required to reveal the copepod diversity of Costa Rican freshwater systems. New inland aquatic habitats should be sampled, like ephemeral pools, subterranean waters, caves, methane seeps, and even semi-terrestrial habitats, often harboring a rich copepod fauna (Reid 1986).

A consistent plan to develop more human resources formed in the taxonomy and systematics of these microcrustaceans is required as a basic strategy to increase the knowledge of this biodiversity. Thereby we could increase the knowledge of our biological diversity and thus allow the development of improved conservation strategies (Mercado-Salas et al. 2013) and prevent or mitigate some of the problems related to the loss of biodiversity (Molnar et al. 2008).

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A new species of the genus *Nihonotrypaea* Manning & Tamaki, 1998 (Crustacea, Decapoda, Axiidea, Callianassidae) from the South China Sea

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Abstract

A new species of the genus *Nihonotrypaea* Manning & Tamaki, 1998, *N. hainanensis* **sp. n.**, collected from the South China Sea, is described and illustrated. It is distinguishable from *N. harmandi* (Bouvier, 1901), *N. japonica* (Ortmann, 1891), *N. thermophila* Lin, Komai & Chan, 2007 and *N. makarovi* Martin, 2013 by having the elongated carpus of the male and female major cheliped. The new species is distinguishable from *N. petalura* (Stimpson, 1860) by the proximolower margin of the carpus of the male major cheliped bearing several small denticles.

Keywords

Callianassidae, *Nihonotrypaea*, new species, South China Sea

Introduction

While working on the taxonomic study of the axiidean fauna (Crustacea, Decapoda) of the China Sea, an undescribed species assignable to the genus *Nihonotrypaea* Manning & Tamaki, 1998 was found from the intertidal sand flat of Hainan Province, South China Sea. The genus *Nihonotrypaea* is characterized by the following characters: carapace lacking rostral spine, minute median terminal spinule present or absent; antennular and antennal peduncles subequal in length; third maxilliped lacking exopod, ischium-merus operculiform, distal margin of merus slightly projecting beyond articulation with carpus; chelipeds unequal, both with lobe-like protrusions on lower margin of merus; first pleopod slender, uniramous in both sexes; second pleopod absent in male, slender, biramous in female; third to fifth pleopods with stubby, slightly projecting appendix internae in both sexes (Manning and Tamaki 1998, Lin et al. 2007).

Five species are known, all from the northwestern Pacific: *N. harmandi* (Bouvier, 1901), *N. japonica* (Ortmann, 1891), *N. petalura* (Stimpson, 1860), *N. thermophila* Lin, Komai & Chan, 2007 and *N. makarovi* Martin, 2013. The status of the genus has been subject to disagreement. Sakai (1999, 2005) treated *Nihonotrypaea* as a synonym of *Callianassa* Leach, 1814. Subsequently, Sakai (2011) redefined several genera belonging to the subfamily Callianassinae, and placed *Nihonotrypaea* under the synonymy of *Trypaea* Dana, 1852. On the other hand, *Nihonotrypaea* was recognized as a valid genus by Tudge et al. (2000), Lin et al. (2007) and Martin (2013). Preliminary molecular phylogenetic analyses of the family Callianassidae (Felder and Robles 2009) showed that the representatives of the genus *Nihonotrypaea* grouped together with those of the genus *Neotrypaea* in a strongly supported monophyletic clade, encompassing somewhat less supported subclades that do not clearly resolve the status of *Nihonotrypaea*. Meanwhile, the specific status of *N. harmandi* has been subject of debate: for example, Tamaki et al. (1997, 1999), Tamaki and Miyabe (2000), Wardiatno and Tamaki (2001), Tamaki and Harada (2006) and Lin et al. (2007) considered it a valid species. On the other hand, Sakai (1969, 1987, 1999, 2004, 2011) considered it as a junior synonym of *Nihonotrypaea japonica* or *Trypaea japonica*.

In this study, we provisionally recognize the genus *Nihonotrypaea* according to the latest literature (Martin 2013), and the higher classification of Callianassidae is not discussed in depth, as it is beyond the scope of this paper. Here we describe and illustrate a new species, referred to *Nihonotrypaea* from the Chinese seas.

Methods

All specimens examined have been deposited in the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China (IOCAS). The drawings were made with the aid of drawing tube mounted on a Zeiss Stemi Sv11 compound microscope. The following abbreviation is used throughout the text: CL: carapace length.

Taxonomy

Family Callianassidae Dana, 1852
Genus *Nihonotrypaea* Manning & Tamaki, 1998

Nihonotrypaea hainanensis sp. n.

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Figs 1–3

Material examined. Holotype, adult male (CL 2.9 mm), MBM136863, Sanya City, Hainan Province, South China Sea, 18°17.2'N, 109°26.4'E, intertidal zone, sand, coll. Fengxuan Zhang, 5 April 1958. Paratypes, 2 ovig. females (CL 3.0, 3.2 mm), MBM136863, same data as holotype.

Description. Rostrum (Fig. 1A, B) broadly triangular, obtuse, directed slightly downwards, reaching proximal 0.2 length of eyestalk in dorsal view. Carapace smooth (Fig. 1A, B), approximately 0.3 of total body length; dorsal oval well defined, 0.74 as long as carapace. Cervical groove located at posterior quarter; linea thalassinica complete.

Eyestalks (Fig. 1A, B) moderately long, sub-triangular in dorsal view, lateral margin distinctly sinuous, anterolateral margin forming thin ridge extending to rounded terminal margin, reaching 4/5 length of first article of antennular peduncle; cornea subterminal, disk-shaped, swollen in lateral view, with scattered brown-pigmented spots, corneal width less than half of basal width of eyestalk.

Antennular peduncle (Fig. 1A, B) subequal in length to antennal peduncle; third article 2.4 times as long as second article; outer flagellum and inner flagellum shorter than peduncle. Antennal peduncle with rudimentary scale on article 3; fifth article distinctly shorter than fourth article.

Third maxilliped (Fig. 2A, B) without exopod; ischium-merus operculiform, with dense setae on ventral margin; ischium broader than long, inner surface with crista dentata consisting of slightly sinuous row of small and sharp denticles; merus broader than long and approximately 0.7 times as long as ischium; carpus cup-shaped, shorter than merus; propodus slender, almost as long as carpus; dactylus moderately slender, digitiform, shorter than propodus. Inner surface of merus to dactylus with blunt longitudinal ridge bearing rows of long setae.

Pereopods 1 unequal and strongly dissimilar. Male major cheliped massive (Fig. 2C). Ischium 3.0 times as long as high; upper margin sinuous, with 1 small denticle proximally; lower margin slightly convex, armed with 4 inconspicuous denticles in distal 0.3. Merus as long as ischium, 1.9 times as long as high; upper margin slightly convex, unarmed; lower margin sinuous, with prominent lobe-like process proximally and 2 minute, inconspicuous denticles subdistally; lobe-like process terminating in acutely, with 7 sharp denticles except for the tip on ventral margin and 1 subterminal denticle distally. Carpus elongated, 1.7 times as long as high, 1.3 times as long as merus; upper margin almost straight; proximolower margin gently convex, with row of several small,

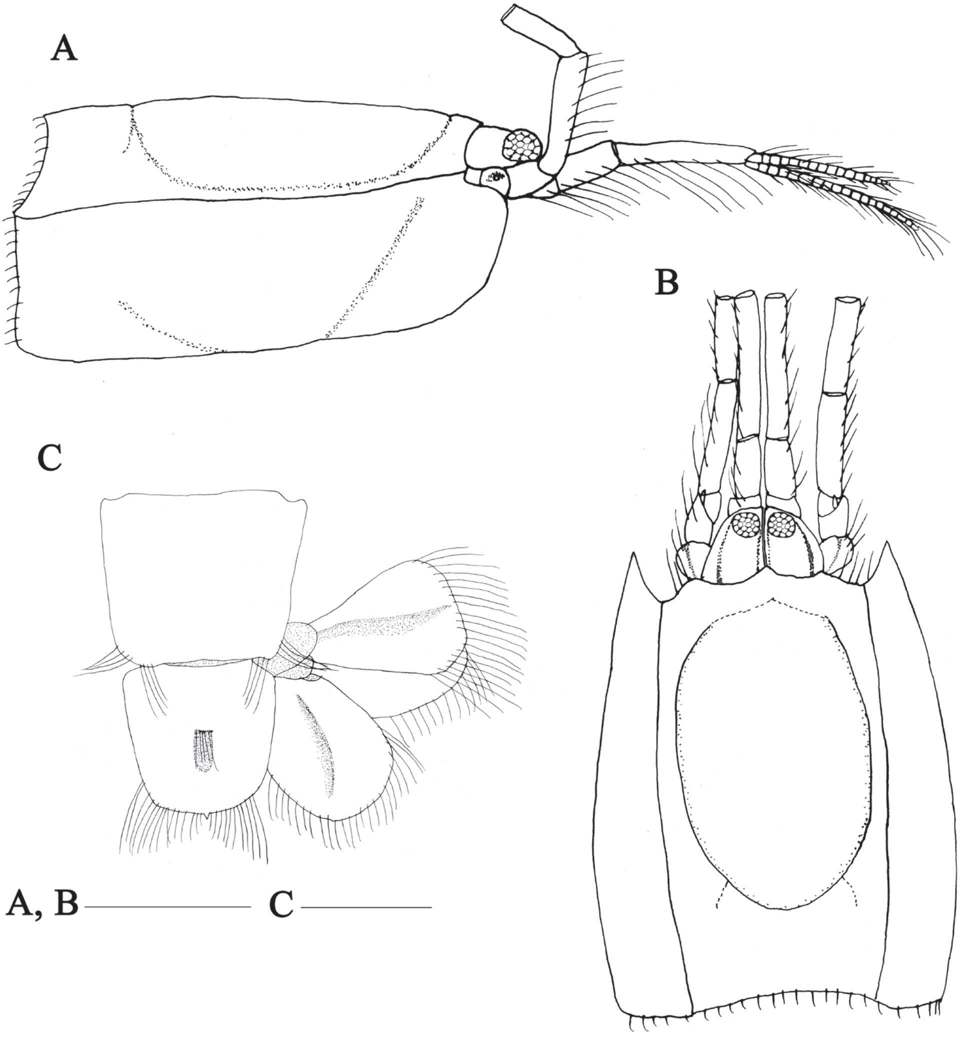

Figure 1. *Nihonotrypaea hainanensis* sp. n. Holotype male, MBM136863. **A–B** carapace, dorsal and lateral view **C** pleomere 6, telson and uropods, dorsal view. Scale = 1 mm.

blunt denticles. Chela heavy, approximately 1.6 times as long as high; palm subquadrate, approximately 1.1 times as long as high, 0.6 times as long as carpus; fixed finger 0.5 times as long as palm, cutting edge with some inconspicuous denticles in distal 0.3; dactylus slightly curved distally, slightly longer than fixed finger, cutting edge sinuous, entire in the distal 0.2 and proximal 0.3, and armed with row of denticles on middle.

Male minor cheliped (Fig. 2D) with ischium 3.5 times as long as high, unarmed. Merus 0.9 times as long as ischium, about 2.3 times as long as high, upper and lower margin slightly convex, unarmed; outer surface medially swollen. Carpus 2.5 times as long as high, 1.6 times as long as merus, abruptly narrowed at base; upper margin almost straight; proximolower margin convex. Chela gapped between slender dactylus

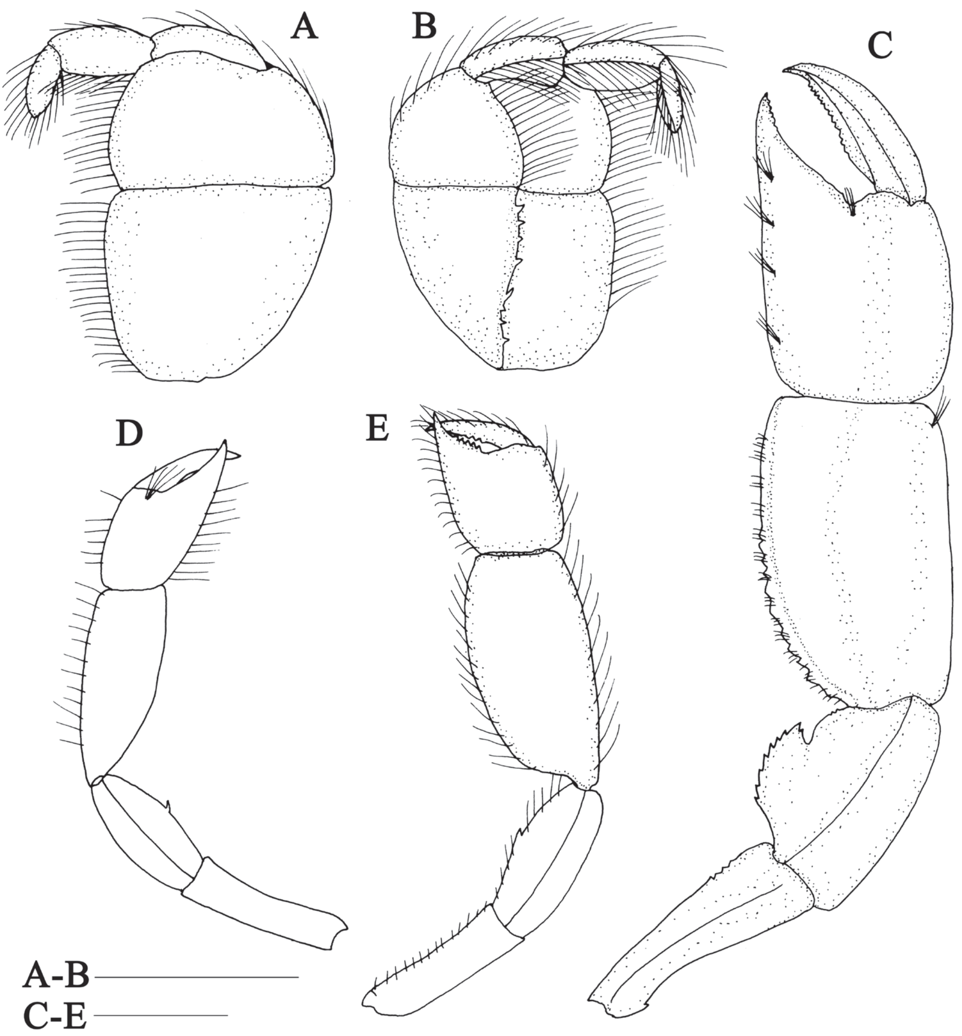

Figure 2. *Nihonotrypaea hainanensis* sp. n. **A–D** Holotype male, MBM136863 **E** Paratype female, MBM136863. **A–B** maxilliped 3, outer and inner views **C** male left major cheliped, outer view **D** male right minor cheliped, outer view **E** female left major cheliped, outer view. Scale = 1 mm.

and fixed finger; palm nearly as long as high, 0.5 times as long as carpus; fixed finger tapering distally to acute tip, cutting edge unarmed; dactylus slightly longer as palm, slender, unarmed on concave cutting edge.

Female major cheliped (Fig. 2E) with ischium 3.5 times as long as high, upper margin almost straight; lower margin slightly concave, unarmed. Merus almost as long as ischium, upper margin slightly convex, unarmed; lower margin slightly convex, with small tooth at midlength. Carpus 2.0 times as long as high, 1.4 times as long as merus; upper margin almost straight. Chela similar to that of male but relatively smaller; palm subquadrate, almost as long as high; fixed finger 0.5 times as long as palm, cutting edge with some small denticles on proximal half; dactylus slightly longer than fixed finger, cutting edge sinuate and with some small denticles on middle. Minor cheliped in female similar to that of male, and about 0.8 times as long as major cheliped.

Pereopod 2 (Fig. 3A) chelate. Ischium 2.0 times as long as high; merus about 3.0 times as long as high, upper margin smooth, lower margin protruding and with row of dense long setae; carpus subtriangular, shorter than merus; chela slightly shorter than carpus, with dense setae on lower and upper margins; palm with upper margin slightly convex; dactylus 1.9 times as long as upper margin of palm; carpus and chela fringed with short to long setae along margins.

Pereopod 3 (Fig. 3B) simple, moderately slender. Ischium slender, approximately 2.0 times as long as high; merus approximately 2.7 times as long as high; carpus subtriangular, shorter and broader than merus, broadest subdistally, approximately 1.9 times as long as high; propodus subrectangular, lower margin roundly swollen, upper margin slightly convex and 0.6 length of carpus, with numerous tufts of setae on lateral surface and row of thick setae along upper and lower margin, no distinct heel delimited; dactylus subtriangular, upper and lower margin convex, outer surface densely setose, terminating in corneous tip.

Pereopod 4 (Fig. 3C) slender, all articles unarmed. Ischium rectangular; merus slightly longer than ischium; carpus 0.6 length of merus; propodus 2.8 times as long as carpus, lower margin densely setose; dactylus tapering distally, setose on lateral margin.

Pereopod 5 (Fig. 3D) slender, semichelate, all articles unarmed. Ischium rectangular; merus about 2.5 as long as ischium; carpus about 0.6 length of merus, upper margin swollen; propodus about 1.3 times as long as carpus, lower distal corner projecting to form a chela with dactylus, lateral surface beset distally with dense setae; dactylus hooked toward external side of fixed finger, tips of dactylus and fixed finger obtuse.

Pleomeres smooth dorsally. Pleomere 1 narrowing anteriorly in dorsal view; dorsal tergite fused with the lateral pleurites; pleuron weakly developed but with clearly defined ventral margin. Pleomere 2 distinctly longer than other pleomeres, with posterolateral margin of pleuron slightly expanded, bearing lateral row of plumose setae. Pleomere 3–5 with pleura each having tuft of moderately long plumose setae. Pleomere 6 (Fig. 1C) subquadrate in dorsal view, very slightly narrowed posteriorly; lateral margin smooth, without conspicuous notch.

Telson (Fig. 1C) trapezoidal, almost as long as wide and 0.8 times as long as pleomere 6; dorsal surface convex with a short transverse row of setae medially; lateral margin unarmed; posterior margin gently convex, with small median spine.

Male pleopod 1 (Fig. 3E) uniramous, 2-articulated; distal article with some distal setae. Male pleopod 2 absent. Female pleopod 1 (Fig. 3F) uniramous, 2-articulated; protopod article sinuous, shorter than ramus; ramus spatulate distally and weakly thickened basally, bearing short setae on both margins. Female pleopod 2 (Fig. 3G) biramous; exopod 2-articulated, approximately 1.5 times as long as endopod, sinuous, bearing some short setae distally; endopod with long setae on proximal part and short setae terminally. Pleopods 3–5 biramous, foliaceous, rami broad; appendix internae (Fig. 3H) stubby, slightly projecting beyond margin of endopod, bearing numerous small adhesive hooks along mesial margin.

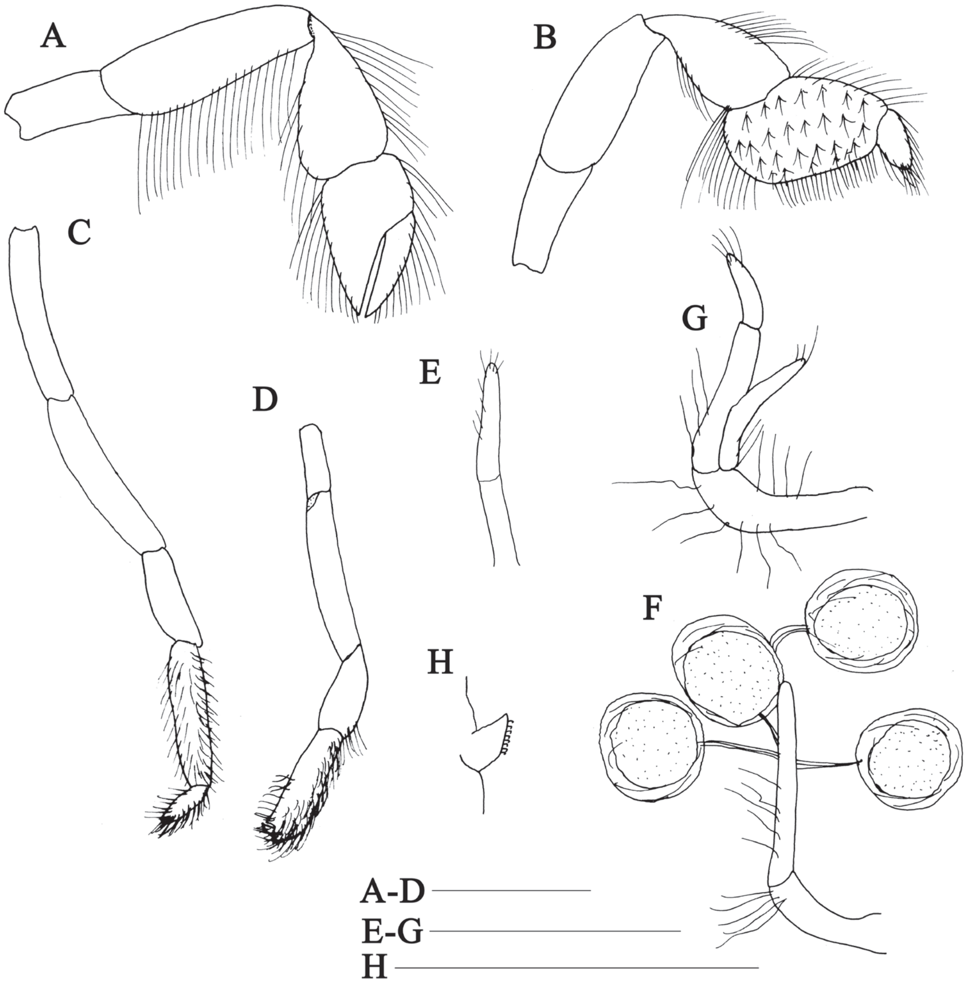

Figure 3. *Nihonotrypaea hainanensis* sp. n. **A–E** Holotype male, MBM136863 **F–H** Paratype female, MBM136863 **A–D** pereopods 2–5, outer views **E** male pleopod 1, posterior view **F** female pleopod 1, posterior view **G** female pleopod 2, posterior view **H** appendix internae of pleopod 3, posterior view. Scale = 1 mm.

Uropodal endopod (Fig. 1C) subovate, slightly longer than telson, 1.5 times as long as wide; margins unarmed; with distinct submedian carina on dorsal surface. Uropodal exopod (Fig. 1C) broadened and fan-shaped, almost as long as wide; margins unarmed, with a distinct submedian carina on dorsal surface.

Remarks. *Nihonotrypaea hainanensis* sp. n. is the sixth species assigned to the genus. The new species is closely related to *N. harmandi* (Bouvier, 1901), *N. japonica* (Ortmann, 1891) and *N. petalura* (Stimpson, 1860) in the antennular peduncle being subequal in the length to the antennal peduncle, whereas *N. thermophila* Lin, Komai & Chan, 2007 and *N. makarovi* Martin, 2013 have the antennular peduncle being slightly to distinctly shorter than the antennal peduncle. *Nihonotrypaea hainanensis* sp. n. is distinguishable from *N. harmandi* (Bouvier, 1901), *N. japonica* (Ortmann, 1891), *N. thermophila* Lin, Komai & Chan, 2007 and *N. makarovi* Martin, 2013 by having a relatively long carpus (approximately 1.7 times as long as high in male and 2.0 times as long as high in female) of the major cheliped (versus carpus subquadrate, in male, approximately 1.1 times as long as high in the later four species; in female approximately 1.5 time as long as high in *N. makarovi*, 1.1 times as long as high in *N. harmandi*, *N. japonica* and *N. thermophila*). Sakai (1969) discussed the variation of the major cheliped in *N. petalura*: some specimen have the oblong carpus (approximately 1.5–1.8 times as long as high), which is similar to *Nihonotrypaea hainanensis* sp. n., but the new species can be readily distinguished from *N. petalura* by the proximolower margin of the carpus of the major cheliped bearing several small denticles (versus smooth in *N. petalura*).

It is worth mentioning that considerable similarities were also found between species assigned to *Nihonotrypaea* and those of *Biffarius* Manning & Felder, 1991, especially regarding operculiform ischium-mems on the third maxilliped. The new species is also closely related to *B. ceramicus* (Fulton & Grant, 1906) and *B. melissae* Poore, 2008 in having a broadly triangular rostrum and the antennular peduncle being subequal in length to the antennal peduncle. It is, however, distinguishable from *B. melissae* Poore, 2008 by having the telson almost as long as wide (telson about 0.8 times as long as wide), and can be distinguished from *B. ceramicus* (Fulton & Grant, 1906) by having a relatively long carpus of the male major cheliped (approximately 0.8 times as long as high).

Etymology. The species name is based on the type locality, Hainan Province of China.

Distribution and habitat. Presently only known from the type locality. Found in the intertidal zone in sand.

Acknowledgments

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Crustaceans from antipatharians on banks of the northwestern Gulf of Mexico

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Abstract

The stalked barnacle *Oxynaspis gracilis*, the chirostylid squat lobster *Uroptychus* sp., and the caridean shrimps *Periclimenes* cf. *antipathophilus* and *Pseudopontonides principis* have been collected at 68–124 m by a remotely operated vehicle (ROV) on banks in the northern Gulf of Mexico. These species inhabited six species of antipatharian hosts. *Pseudopontonides principis*, *O. gracilis*, and *U.* sp. were not confined to a single host species. Except for *O. gracilis*, collected by ROV in 2004–2005, these species have not been reported previously in the northwestern Gulf of Mexico.

Keywords

Anomura, Caridea, Cirripedia, Antipatharia, Gulf of Mexico

Introduction

Antipatharians (Cnidaria: Anthozoa: Hexacorallia), commonly called black corals or wire corals, tend to inhabit deeper reef areas or cliffs in the greater Caribbean-west Atlantic region (Fig. 1). In the Gulf of Mexico, they rarely inhabit depths that can be reached by SCUBA divers. Boland (2005) reported *Plumapathes pennacea* (Pallas) from four oil platforms at 9.5-43.0 m at the following locations: 28° 00'N, 93°17'W; 27°8'N, 93°40'W; 27°46'N, 93°8'W; 27°46'N, 93°19'W; and at the East Flower Garden Bank buoy 6, 27°55'N, 93°36'W. One of us (MW) saw (but did not collect or photograph) a "wire coral" (*Stichopathes* sp.) at a depth of 33 m on West Flower Garden Bank (27°52.5'N, 93°49'W), 2 Sept. 2004.

Black corals have been documented to host many associated species, including crustaceans, polychaetes, mollusks, ophiuroids, and fishes (Totton 1923, Warner 1981, Humes 1990, Grange 1991, Pettibone 1991, Glasby 1994, Wirtz and d'Udekem-d'Acoz 2001, Castro et al. 2004, Molodtsova and Budaeva 2007, Tazioli et al. 2007). We have not found any records of antipatharian-associated fauna in the northwestern Gulf of Mexico. We here present records, depths and species associations of a cirripede and three decapod crustaceans that associate with black corals.

Methods

MN collected specimens in the vicinity of the East and West Flower Garden Banks, Horseshoe Bank, 29-Fathom Bank, Rankin Bank, 28-Fathom Bank, Bright Bank, and Geyer Bank at depths ranging from 68.0 to 123.9 in 2011–2012 (Table 1). Figure 1 shows the natural habitat. Specimens were retrieved using a single function manipulator on a Phantom S2 remotely operated vehicle (ROV). When associated fauna were found, they were removed carefully from the black coral, photographed, and placed in separate containers with 95% ethanol. The photographs of the freshly collected shrimps are not sufficiently detailed for publication, but a photograph of *Uroptychus* sp., although the specimen was broken, is of sufficient detail to show the living color pattern.

Antipatharian samples were identified using traditional morphological techniques (corallum branching mode, subpinnule branching patterns, spine morphology and size, and polyp size and distribution). Tissue was removed from a small section of each colony using a 50/50 sodium hypochlorite water solution and an ultrasonic cleaner, dried, and coated with gold-palladium using a sputter coater (70 mm target distance, 30 mA, 30 secs) to obtain scanning electron micrographs using a Hitachi TM3000 tabletop scanning electron microscope for analysis of skeletal morphology. MN identified specimens, with problematic species identifications confirmed by black coral taxonomist Dennis Opresko, Research Associate, United States National Museum of Natural History. Antipatharians recently have undergone taxonomic revision (Opresko 1972, 1974, 2001, 2004; Perez et al. 2005). We used currently accepted names when we were sure of the identification. Taxonomic confusion remains regarding the differences (if any) between *Antipathes atlantica* Gray and *A. gracilis* Gray, hence in our records we record these species as *Antipathes atlantica/gracilis*. See Nuttall (2013) for further information on methods and the scope of the projects involved during collection.

Records of the material examined are given in Table 1. Host antipatharians species are not reported for many of the shrimps because they were loose from their hosts after

Table 1. Material examined

Coordinates	**Collection number**	**Depth, date**	**Number, host**
Oxynaspis gracilis:			
East Flower Garden Bank:			
27°57.2'N, 93°36.0'W	DFH8-19A14, TCWC 2-8958	60 m, 3 Sept. 2004	2, on *Antipathes atlantica/gracilis*
27°57'N, 93°36'W	DFH11-3A, TCWC 2-9121	99 m, 13 Sept. 2005	3, on "*Antipathes* sp."
West Flower Garden Bank:			
27°51.2'N, 93°49.2'W	DFH17-17C, TCWC 2-3663	82.4 m, 1 Aug. 2012	1, on *Phanopathes expansa*
27°53.9'N, 93°47.0'W	DFH17-25B, TCWC 2-3665	97 m, 1 Aug. 2012	1, on *Elatopathes abientina*
27°56.8'N, 93°37.4'W	DFH17-30C, TCWC 2-3666	87.8 m, 2 Aug. 2012	1, on *Antipathes atlantica/gracilis*
27°53.9'N, 93°27.0 W	PSBF2-18B, TCWC 2-3695	116.4 m, 22 Sept. 2012	2, on *Acanthopathes thyoides*
27°51.1'N, 93°26.3'W	PSBF2-19B, TCWC 2-3696	115.5 m, 22 Sept. 2012	2, on *Acanthopathes thyoides*
27°53.8'N, 93°19.6'W	PSBF3-2D, TCWC 2-3668	86.2 m, 26 Sept. 2012	1, on *Tanacetipathes barbadensis*
27°53.4'N, 93°15.6'W	PSBF3-4B, TCWC 2-3669	123.9 m, 26 Sept. 2012	1, on *Antipathes furcata*
27°52.2'N, 93°17.6'W	PSBF3-8B, not catalogued	89.1 m, 27 Sept. 2012	15, on *Antipathes atlantica/gracilis*
27°50.6'N, 93°3.6'W	PSBF3-12B, not catalogued	90.7 m, 28 Sept. 2012	2, on *Antipathes atlantica/gracilis*
27°50.3'N, 93°3.7'W	PSBF3-16B, TCWC 2-3698	86.2 m, 28 Sept. 2012	4, on *Antipathes atlantica/gracilis*
Horseshoe Bank:			
27°49.9'N, 93°3.37'W	PSBF3-17B, donated to California Academy of Sciences	87.8 m, 28 Sept. 2012	2, on *Antipathes atlantica/gracilis*
27°48.8'N, 93°41.5'W	PSBF1-3B, TCWC 2-2-3694	112.5 m, 24 Oct. 2011	4, on *Phanopathes expansa*
27°52.4'N, 93°42.3'W	PSBF3-10B, TCWC 2-3697	106.1 m, 25 Oct. 2011	2, on *Phanopathes expansa*
29-Fathom Bank:			
28°7.7'N, 93°28.9'W	PSBF1-13C, TCWC 2-3667	68 m, 26 Oct. 2011	1, on *Antipathes atlantica/gracilis*
Uroptychus sp.:			
West Flower Garden Bank:			
27°51.5'N, 93°49.7'W	DFH17-15B, TCWC 2-3636, one donated to U.S. National Museum	78.5 m, 1 Aug. 2012	2, on *Tanacetipathes thamnea*
27°51.3'N, 93°49.6'W	DFH17-16E, TCWC 2-3657	81 m, 1 Aug. 2012	1, on *Tanacetipathes tanacetum*
27°50.1'N, 93°51.3'W	DFH17-22B, TCWC 2-3658	120.3 m, 1 Aug. 2012	1, on *Acanthopathes thyoides*
Rankin Bank:			
27°55.2'N, 93°24.8'W	PSBF2-3C, sent to Kumamoto University	89.3 m, 11 Sept. 2012	2, on *Tanacetipathes* cf. *paula*
Periclimenes cf. *antipathophilus*:			
West Flower Garden Bank:			
27°50.9'N, 93°48.1'W	DFH17-20C, TCWC 2-3646	112.8 m., 1 Aug. 2012	1, on *Acanthopathes thyoides*
Pseudopontonides principis:			
West Flower Garden Bank:			
27°51.5'N, 93°49.7'W	DFH17-15C, TCWC 2-3648	78.5 m. 1 Aug. 2012	1, on *Tanacetipathes thamnea*
27°51.3'N, 93°49.6'W	DFH17-16B, TCWC 2-3649	81 m, 1 Aug. 2012	1, on *Tanacetipathes tanacetum*
27°51.3'N, 93°49.6'W	DFH 17-16C, TCWC 2-3654	81 m, 1 Aug. 2012	1, on *Tanacetipathes tanacetum*
27°51.2'N, 93°49.2'W	DFH 17-17B, TCWC 2-3650	82.4 m, 1 Aug. 2012	1, on *Phanopathes expansa*

Coordinates	Collection number	Depth, date	Number, host
East Flower Garden Bank:			
27°53.3'N, 93°36.8'W	DFH17-33D, TCWC 2-3651	90.9 m. 2 Aug. 2012	4, on *Tanacetipathes thamnea*
27° 53.3'N, 93°36.8'W	DFH17-33C, TCWC 2-3662	90.9 m, 2 Aug. 2012	1, on *Tanacetipathes thamnea*
Bright Bank:			
27°53.8'N, 93°19.6'W	PSBF3-2C, TCWC 2-3653	86.2 m, 26 Sept. 2012	1, on *Tanacetipathes tanacetum*
Rankin Bank:			
27°55.2'N, 93°24.8'W	PSBF2-3C, TCWC 2-3655	89.3 m, 19 Sept. 2012	1, on *Tanacetipathes* cf. *paula*

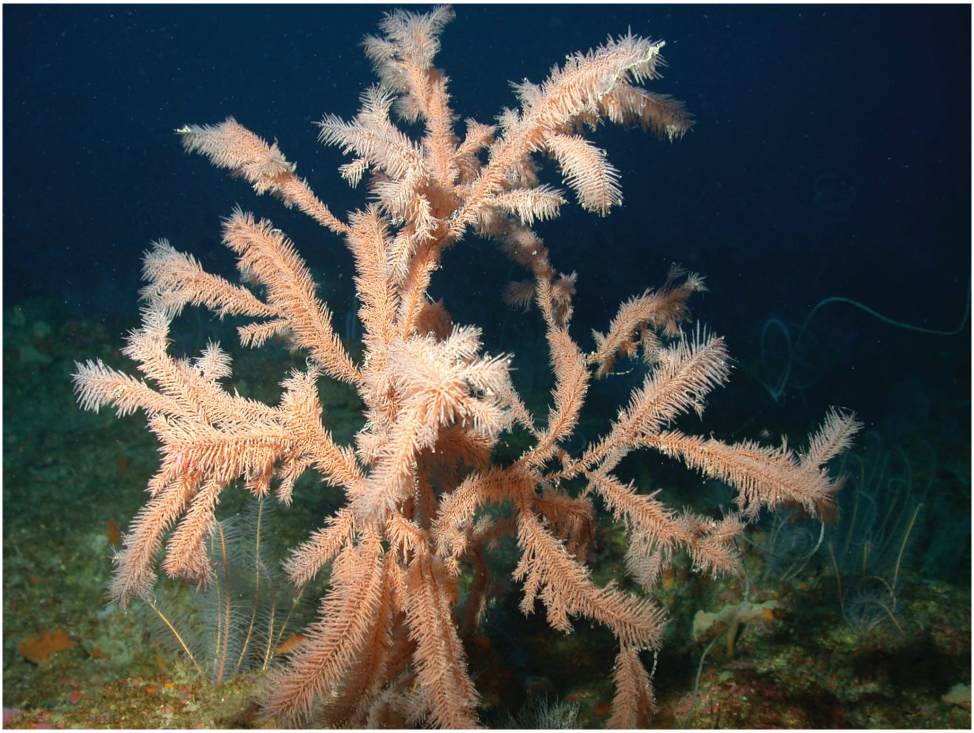

Figure 1. Deep reef habitat showing antipatharians *Tanacetipathes* sp. (foreground) and *Stichopathes* sp. (distant) among protruding arms of comatulid crinoids (West Flower Garden Bank, 80 m).

collection. Because the two species are considered to be obligate associates of antipatharians, we are confident that they were living on antipatharians before collection. MW identified the carideans and most of the cirripedes. Stephen Gittings, United States National Oceanographic and Atmospheric Administration, identified the specimens of *Oxynaspis gracilis* Totten from DFH8-19A14.

Except as noted, the specimens were incorporated into the Biodiversity Research and Teaching Collection at Texas A&M University (formerly the Texas Cooperative Wildlife Collection, TCWC). A few specimens do not have catalog numbers because they are being used for further study. Two specimens of *Uroptychus* sp. were sent to Keiji Baba, Kumamoto University, Japan, for identification, and another specimen was donated to the collections of the U.S. National Museum of Natural History. Although originally thought to belong to *Uroptychus minutus* Benedict, 1902, these specimens now are considered to represent an undescribed species. Two specimens of *Oxynaspis gracilis* were donated to the California Academy of Sciences. The collections were part of the Deep Fish Habitat (DFH) and Potentially Sensitive Biological Features (PSBF) studies of the Flower Gardens Banks National Marine Sanctuary collected under permit number FGBNMS-2009-001.

Results

Oxynaspis gracilis, the black coral barnacle (Cirripedia, Lepadomorpha, Oxynaspididae) is a small (2–5 mm in total length), stalked species found attached to the axes of the corals. It was found on six species, including bottlebrush and fan-shaped species, at 68.0 to 123.9 m. The living tissue of the coral host may encrust the cirriped (Figure 2). This barnacle was collected previously at the East Flower Garden Bank (Table 1). Humann and DeLoach (2002) reported *O. gracilis* as "occasional Caribbean" on *P. pennacea* (Pallas) (as *A. pennacea*) and *A. salix* Pourtalés. (P.A. McLaughlin, Western Washington University verified the identification of the cirripeds in their photograph). Gittings et al. (1986) did not report this species from the northern Gulf of Mexico in their guidebook, nor is it listed in the master crustacean species list for the United States and Canada (McLaughlin et al. 2005).

Uroptychus sp. (Decapoda, Anomura, Chirostylidae) was found clinging to the main axes of *Tanacetipathes* spp. and the "sea fan" antipatharian *Acanthopathes thyoides*, (Pourtalés) at 78.5–120.3 m. Photographs of a freshly collected individual show that the cephalothorax was red, and the chelipeds translucent with a red lateral stripe on the propodus and carpus and red spots on the palm of the chela (Figure 3). An as yet unidentified specimen of *Uroptychus* sp. from southwestern Florida has a similar color pattern (D. Felder, pers. comm.)

Two shrimps (Decapoda, Caridea, Palaemonidae) were found on the antipatharians. *Pseudopontonides principis* (Criales), the wire coral shrimp, previously has been reported from the northeastern Gulf of Mexico and Curacao, Bonaire, and Puerto Rico in the Caribbean (Heard 1986, Humann and DeLoach 2002) on *Stichopathes leutkeni* (Brook) (as *Cirrhipathes leutkeni*), *A. gracilis*, *P. pennacea*, and *Virgularia* sp. (Pennatulacea). In the northwestern Gulf of Mexico, it has been found on *Tanacetipathes* spp. and *Phanopathes expansa* (Opresko and Cairns 1992).

The other caridean, found on *Acanthopathes thyoides*, is broken and cannot be identified definitely to species. The form of the pereopods, first antennae and rostrum are consistent with *Periclimenes antipathophilus* Spotte, Heard & Bubucis, 1994, the black coral shrimp. A photograph of our freshly collected specimen has the same color pattern as that photographed by Humann and DeLoach (2002). This species has been reported in the Bahamas, Turks and Caicos Islands, and eastern Honduras in the Caribbean on *Stichopathes gracilis* and *Stichopathes* sp. (as *Cirrhipathes* sp). It also is common at Bonaire, Netherlands Antilles (R. Heard, pers. comm.)

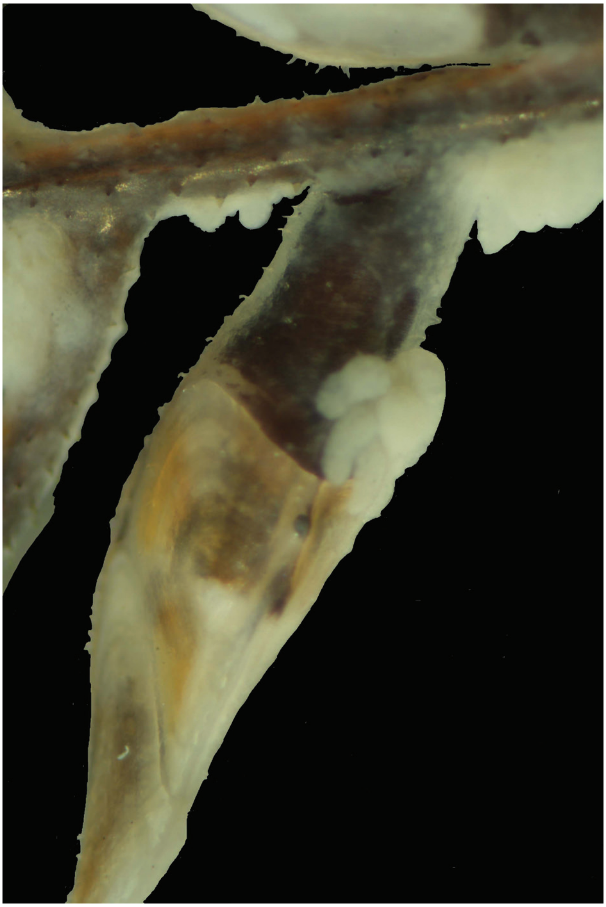

Figure 2. *Antipathes atlantica/gracilis* overgrowing *Oxynaspis gracilis.* (Horseshoe Bank, 112 m, sta. PSBF1-3B).

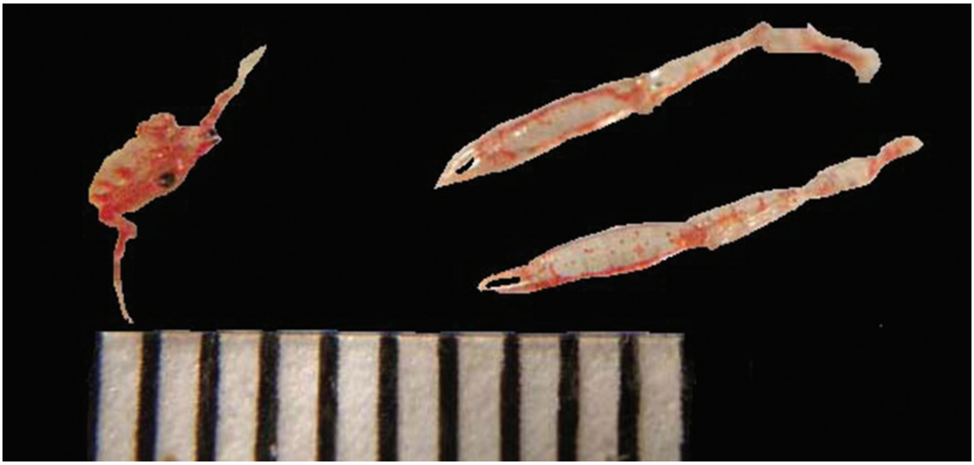

Figure 3. *Uroptychus* sp., freshly caught (West Flower Garden Bank, 79 m). Scale in mm.

Also found on the antipatharians was the wing oyster *Pteria colymbus* (Röding). The wing oyster commonly occurs on gorgonians, antipatharians, or other invertebrates that project upward from the surface of reefs and is not considered to be a strict associate of antipatharians.

Multiple species of associated fauna were documented on a single black coral host. Both *U. minutus* and *Pseudopontonides principis* were found on the bottle brush shaped antipatharians *Tanacetipathes* sp. Both *P. principis* and *O. gracilis* lived on sea fan antipatharian *P. expansa*.

Discussion

Wirtz and d'Udekem-d'Acoz (2001) collected seven species of decapods from antipatharians and gorgonians at the Cape Verde Islands, none of them the same species as found in the Gulf of Mexico. These collections were made by SCUBA diving at depths of 15-30 m, considerably shallow in comparison to our collections. Of the seven species, three were found only on antipatharians, and one shrimp, *Periclimenes wirtzi* d'Udekem d'Acoz might be an obligate associate of antipatharians. Previous reports of *P. antipathophilus* indicate that it, too, is only found on antipatharians. The authors reported that one shrimp, *Pseudocoutierea wirtzi* d'Udekem d'Acoz lived in groups of "many individuals" on gorgonians, but only solitary shrimp of this species were found on antipatharians. Because our specimens were collected by ROV, we cannot be sure if *Pseudopontonides principis* occurred in "groups" but one sample contained four individuals. Two or three individuals of *P. principis*, sometimes of different color patterns, have been photographed on antipatharians in the Caribbean (L. Wilk, pers.comm.). The cirripede occurred singly or in clusters of as many as 15 individuals.

To collect and observe the minute associated crustaceans of colonial cnidarians, one cannot use trawls or dredges. The smaller specimens slip through the mesh or are smashed and unrecognizable. Although the Phantom S2 is equipped with both video and still cameras, it cannot approach a black coral closely enough to see the tiny associated crustaceans. Additionally, the inability to document these interactions in situ makes defining the nature of the relationships between the associated fauna and the host difficult to determine. Although the presence of associated fauna has been documented to alter corallum or spinal morphology of the black coral host (Molodtsova and Budaeva 2007), the host fauna in this study appeared healthy and unmodified, with the exception of black coral overgrowth of *O. gracilis*.

The majority of antipatharians in the northern Gulf of Mexico live in mesophotic or deep-sea environments, beyond the depth range of conventional SCUBA diving. Data from previous collections of antipatharians suggest that they inhabit many banks in the northern Gulf of Mexico. With proper collecting technique, it is likely that investigators will find additional specimens of the associated crustaceans and perhaps additional species.

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Grooming as a secondary behavior in the shrimp *Macrobrachium rosenbergii* (Crustacea, Decapoda, Caridea)

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Abstract

The giant freshwater prawn, *Macrobrachium rosenbergii*, is a large shrimp extensively used in aquaculture whose grooming behaviors were analyzed in this study. *Macrobrachium rosenbergii* exhibits three unique male morphotypes that differ in their behavior, morphology and physiology: small-clawed males (SM), orange-clawed males (OC) and blue-clawed males (BC). The largest and most dominant males, BC males, are predicted to have significantly different grooming behaviors compared to females and the other two male morphotypes. These BC males may be too large and bulky to efficiently groom and may dedicate more time to mating and agonistic interactions than grooming behaviors. Observations were conducted to look at the prevalence of grooming behaviors in the absence and presence of conspecifics and to determine if any differences in grooming behavior exist among the sexes and male morphotypes. Significant differences in the grooming behaviors of all individuals (females and male morphotypes) were found. BC males tended to have the highest grooming time budget (percent of time spent grooming) while SM males had a relatively low grooming time budget. The grooming behaviors of the male morphotypes differed, indicating while these males play distinct, separate roles in the social hierarchy, they also have different grooming priorities. The conditions in which *M. rosenbergii* are cultured may result in increased body fouling, which may vary, depending on the grooming efficiencies and priorities of these male morphotypes. Overall, grooming behaviors were found to be a secondary behavior which only occurred when primary behaviors such as mating, feeding or fighting were not present.

Keywords

Grooming, aquaculture, *Macrobrachium rosenbergii*

Introduction

Behavioral hierarchy

A behavioral hierarchy occurs among certain behaviors which are deemed essential to an organism. Ranking of behaviors by individuals is necessary when an organism is in conflict situations such as foraging (Davis et al. 1974), fighting a predator or conspecific (Karplus et al. 1987), or mating (Liske and Davis 1986). These behaviors are normally deemed primary behaviors and are usually considered high in a behavioral hierarchy as they are evolutionarily important for reproductive fitness and survival situations. Secondary behaviors should occur when primary behaviors are not critical. An example of a secondary behavior is body grooming which is a behavioral adaptation to fouling pressures. Grooming removes fouling agents and has been hypothesized, but not tested, to be a secondary behavior (Bauer 1989). These secondary behaviors may be inhibited when more vital behaviors are beneficial to survival. Therefore, grooming would decrease when primary behaviors are more pressing (Bauer 1989, 2013).

The environment that an organism inhabits is also an important factor in the behavioral decision making process. Organisms in resource-limiting environments or social situations (i.e. competition, mating, agonistic interactions) should prioritize those behaviors with the greatest resource profitability (primary behaviors, i.e. searching for food or mates) before other subordinate behaviors (Brown 1986, Shettleworth 2009). Thus, it is hypothesized that only when the organism is in a situation where environmental pressures are not critical will secondary behaviors like grooming take place (Bauer 1989). Organisms adjust their behavioral schedule to best accommodate their needs. Grooming is important in daily maintenance activities of organisms to ensure that the body is free of fouling that may impede important primary actions such as foraging for food, reproduction, and avoiding predation (VanMaurik and Wortham 2011).

Grooming behaviors in animals

Grooming is a behavior for removing fouling debris and organisms from body surfaces (Bauer 1977, Felgenhauer and Schram 1978). It is a common behavior seen in many animals including primates (Dunbar 1996), birds (Cotgreave and Clayton 1994), fish (Bshary and Schaffer 2002), insects (Hlavac 1975) and crustaceans (Bauer 1977, 2004, 2013, Felgenhauer and Schram 1978, 1979, Martin and Felgenhauer 1986). Although grooming occurs in both terrestrial and aquatic systems, the former have relatively fewer fouling pressures than the latter (Holmquist 1985). Aquatic biota are constantly bathed in a water medium where fouling can be particularly detrimental if the surrounding water is laden with sediment or fouling organisms (Bauer 2004).

Grooming in Crustaceans

Autogrooming in crustaceans is important for removing macro- and microscopic fouling organisms, debris, sedimentation, and algae from body surfaces (Bauer 1981). Grooming in crustaceans prevents fouling of structures utilized in reproduction (pleopods, Bauer 1979), respiration (gills, Bauer 1998, 1999), sensory reception (antennae; Bauer 1977, 1978), as well as displays and movements by jointed appendages (Bauer 1981, 1989). Fouling of these structures may result in decreased efficiency of respiration and sensory reception along with decreased ability to mate, brood offspring and fight competitors (Bauer 1977, 1978, 1979, 1998, 1999). Crustaceans have an exoskeleton, jointed appendages and soft body parts (i.e. gills) that are periodically molted which can remove some fouling from the outer body surfaces. Although molting removes most fouling agents from the body, the intermolt period may be lengthy, especially for older individuals that are normally larger and molt less frequently and therefore have a decreased ability to rid themselves of fouling (Skinner et al. 1985).

Grooming behaviors have been studied for many crustacean groups, especially in decapod crustaceans such as penaeid and caridean shrimps (Bauer 1977, 1978, 1979, 1981, 1989, 1999, 2004, 2013, Felgenhauer and Schram 1978, 1979, VanMaurik and Wortham 2011), brachyuran crabs (Bauer 1981, Pearson and Olla 1977), anomuran crabs (Martin and Felgenhauer 1986), lobsters (Schmidt and Derby 2005), crayfishes (Bauer 1998, 2002), and stomatopods (Wortham 2008). Despite the phylogenetic relatedness of these groups, there is considerable variability in the grooming behaviors and morphology of decapods. This observed behavioral variation in decapods (crabs, shrimps, lobsters) is thought to accommodate their unique morphologies. As a result, the amount of time devoted to grooming varies among crustacean groups (i.e. grooming time budget).

Study organism

The genus *Macrobrachium* has over 240 species (De Grave et al. 2009, De Grave and Fransen 2011) and has long, robust chelipeds (second pereopods, Fig. 1), especially in large adult males (Wowor et al. 2009). The giant freshwater prawn, *M. rosenbergii* (Crustacea: Decapoda: Caridea), is a caridean shrimp native to rivers of Southeast Asia but has been introduced to most continents for aquaculture. Of the 1.2 million tons of globally cultured shrimps in 2000, only 10% was caridean shrimps. But of that 10%, almost 99.9% were the shrimp, *M. rosenbergii* (FAO 2010), with a global aquaculture economic value of $410 million, representing a significant product (New and Valenti 2000).

Agonistic behaviors and social structure of *Macrobrachium rosenbergii* have been extensively studied due to its use in aquaculture (Barki et al. 1991, Kuris et al. 1987, Ra'anan and Sagi 1985). This species has three distinct male morphotypes, which differ in morphology, physiology and behavior (Ra'anan and Sagi 1985, Kuris et al. 1987, Sagi and Ra'anan 1988) (Figure 2). The smallest males (SM) have small claws

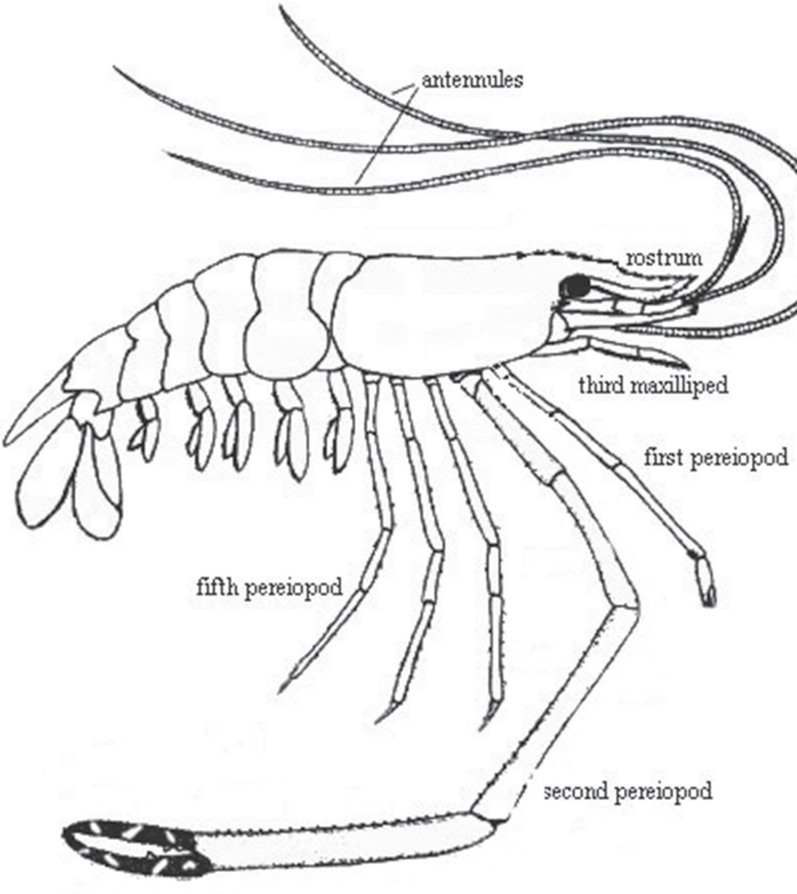

Figure 1. Generalized morphology of *Macrobrachium rosenbergii*. (Diagram modified from Short 2004).

and are subordinate and non-territorial. The intermediate orange-clawed males (OC) are subdominant to the larger males and larger in body size and cheliped (second pereopod) length than SM males. The largest and dominant males in the population are the blue-clawed males (BC). These male morphotypes form a social hierarchy in the population and may be found within the same age class (i.e. all three morphotypes belong to the same cohort) (Kuris et al. 1987, Govind and Pearce 1993).

The three male morphotypes of *M. rosenbergii* exhibit behavioral differences based on their position within the social hierarchy (Kuris et al. 1987). BC males may devote more time to growth of large chelipeds and body size, agonistic interactions with males, reproduction with females, protection of post-molt females, social displays, and defending territories compared to the other male morphotypes. Devotion to these behaviors may result in decreased time available for grooming. While grooming has been a reported behavior for this species, scant information is available (Ra'anan and Sagi 1985, Karplus et al. 1992).

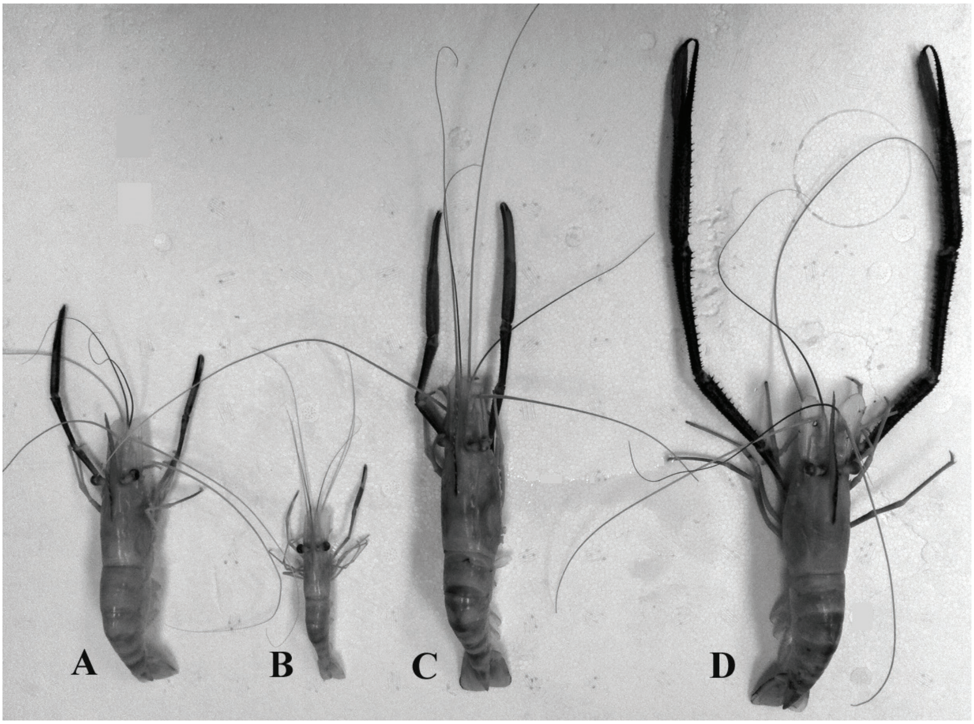

Figure 2. Relative size of *Macrobrachium rosenbergii* female and male morphotypes. **A** Female **B** Small-clawed (SM) male **C** Orange-clawed (OC) male **D** Blue-clawed (BC) male. Note the difference in the size of the chelipeds.

Objectives and hypotheses

Due to the importance of *Macrobrachium rosenbergii* in aquaculture, understanding the grooming behaviors of this species is vital for implementing ways to increase yield and growth by decreasing the potential fouling affecting the morphotypes. The most profitable size is the large BC males and development into this terminal male morphotype depends upon surviving through the SM and OC male phases. We hypothesized *M. rosenbergii* will: (1) have similar grooming behaviors to other caridean shrimps; (2) BC males will have less time available for grooming than other males due to time dedicated to the protection of females, defense of territories and dominance behaviors; and (3) the grooming will be a secondary behavior as hypothesized by Bauer (1989). By studying the grooming behaviors of *M. rosenbergii*, especially the male morphotypes, a key understanding of the tradeoffs between body maintenance and social dominance may be elucidated. Grooming may be an important behavior at the lower levels of the social hierarchy (i.e. SM males) but not in the dominant tier (BC males) where mating and maintaining territories are essential. Understanding how fouling and grooming behaviors vary among morphotypes may impact yield and profit, therefore, aquaculture operators have expressed interest in the grooming behaviors of the morphotypes and the possible ramifications on the survivorship of the shrimps (Craig Upstrom, Aquaculture of Texas, personal communication).

Methods

Macrobrachium rosenbergii were transported overnight from Texas in April 2012 to the University of Tampa. Individual shrimps were added to labeled plastic holding containers (5.5-L), with pre-drilled holes that allowed water flow, and then placed in an 1816-liter fiberglass aquaculture tank with filtered, continuous flowing, aerated water. The individual containers reduced physical contact and agonistic interactions, ensuring that both shrimps' appendages remained intact and death by cannibalism was eliminated. The containers allowed visual and pheromonal contact among individuals as water was able to flow through the pre-drilled holes. The three male morphotypes (SM, OC and BC males) were distinguished by morphological characteristics and correlations among mass and body measurements (Kuris et al. 1987). Shrimps were not fed on testing days; on non-testing days, they were fed shrimp pellets ad libitum. Throughout the study, shrimps were kept on a 14/10 day-night cycle, and water temperature and salinity ranged from 22–24 °C and 5–10 ppt, respectively. Shrimps were not reused in a particular set of observations, however, due to the scarcity and cost of research animals, some shrimps were reused for different sets of observations. Duplicate measurements or observations were never made.

Statistical analyses

The grooming data were analyzed to determine if they met the criteria for parametric statistics. If normality assumptions were not met, then non-parametric statistics were used. Along with variability in individual behavior and failure to meet normality, the grooming data were analyzed using non-parametric statistics. Non-parametric statistical tests used included the Kruskal-Wallis test and the Mann-Whitney U test. Regression analyses were also used. Statistical significance was determined by p-value of less than 0.05.

Grooming Observations

Solitary grooming (Observations #1)

Behavioral observations were conducted to study the grooming behaviors of *Macrobrachium rosenbergii*. The null hypotheses of no difference in relative time budgets allocated to grooming behaviors among the three male morphotypes and between sexes were tested. Individuals were tested in isolation, which helped reduce primary behaviors such as fighting and mating. Each shrimp was used once (N=94) in these solitary observations and placed into a 19-L (40 cm × 25 cm × 20 cm) aquarium with black backing and natural rocky substratum. The black backing ensured that the shrimp would not be influenced by either surrounding shrimps in other tanks or the observer. Water in the aquarium was continually filtered and frequently replaced with water from the aquaculture tanks. Shrimps were allowed to acclimate for 24-hr before testing and were not used if they had molted within seven days. Females with embryos ("eggs") on their pleopods were not observed to control for the behavior among males and females. All grooming behaviors were recorded during the daylight cycle for 30-min using a digital recording device and then later transcribed to data sheets following the methods of VanMaurik and Wortham (2011). These data were used to determine differences in the time spent grooming and body parts groomed among the morphotypes and between the genders.

Social grooming (Observations #2)

The null hypothesis that all behaviors will be equally prioritized was tested. To observe how social interactions with conspecifics affect grooming behaviors, each male morphotype (SM, OC, and BC males) and females were placed in a grooming situation where shrimps could physically touch through antennular and cheliped contact (but not fight) via holes in the individual containers. The objective was to compare grooming behaviors of individuals in an environment without visual or minor physical cues (Solitary Grooming – Observations #1) to that of an environment with visual and physical cues (Social Grooming – Observations #2). These latter observations differed from the Solitary Grooming (Observations #1) because individuals in the isolated situation only had chemical contact through water with other shrimps but did not have visual or minor physical input that was present in the Social Grooming (Observations #2). Visual and minor physical contact with conspecifics was expected to reduce frequency and time allocated to grooming behaviors in these observations, since these behaviors have been predicted to be secondary. Shrimps (N=8; two shrimps of male morphotypes plus females) were observed in the aquaculture tanks in their individual containers for 15-min and their grooming behaviors were recorded. These shrimps were randomly selected from the first observations (Solitary Grooming) and observed 24-hr after being used in the first observations. The same process of recording behaviors was used as in the Solitary Grooming (Observations #1). The data collected in these social observations were extrapolated (multiplied by 2) in order to compare the data to those from the Solitary Grooming (Observations #1) (15-min × 2 = 30-min).

Agonistic interactions (Observations #3)

The null hypothesis that all behaviors are equally prioritized was tested to determine how agonistic interactions (primary behaviors) affect the priority of grooming

Table 1. Experimental design of Agonistic Interactions (Observations #3), listing the ten treatments and the number of trials for each treatment. BC = blue-clawed males; OC = orange-clawed males; SM = small-clawed males; F = females.

Treatment	Individual #1	Individual #2	Sample Size
Treatment 1	BC	BC	2
Treatment 2	BC	OC	2
Treatment 3	BC	SM	2
Treatment 4	BC	F	2
Treatment 5	OC	OC	2
Treatment 6	OC	SM	2
Treatment 7	OC	F	2
Treatment 8	SM	SM	2
Treatment 9	SM	F	2
Treatment 10	F	F	2

behaviors of the male morphotypes (BC, OC, and SM) and females. The objective of these observations was to compare the frequency of grooming behaviors in an environment without visual cues (Solitary Grooming, Observations #1) to that of an environment with physical contact (Agonistic Interactions, Observations #3). If grooming behavior is a secondary action incurring the same energy cost as primary behavior (i.e. mating, fighting, displaying), grooming behaviors should be reduced in time and frequency during these observations compared to both the solitary and social observations (Observations #1 and #2, respectively). During these observations shrimps had physical contact with another individual in a test arena and their grooming behaviors were recorded along with all other behaviors such as swimming, antennular touching, mating, fighting and non-agonistic interactions (interactions with no aggressive behaviors). This is different from the Social Grooming (Observations #2) due to the increased level of interaction (i.e. fighting, mating possible). In Observations #3, shrimps were paired based on morphotype and sex for a total of ten different treatments; there were two trials of each treatment for a total of N=20 observations (Table 1). Following a 24-hr acclimation period within individual containers, shrimps were allowed to acclimate for 30-min in a test arena (within water table: 58 cm × 41 cm × 23 cm) without any contact. For the subsequent 30-min, the shrimp's behaviors were recorded while in it's individual container. Most shrimps had ample room to walk around within its individual container. After the observations were completed, the number of behaviors and types of behaviors were tabulated. Shrimps used in this Agonistic Interaction (Observations #3), were randomly selected from the Solitary Grooming (Observations #1) and were observed after a minimum of 24-hr acclimation period within individual containers.

Results

Grooming observations

Solitary grooming observations (Observations #1)
Appendages: overall

Four appendages were observed actively grooming the body: third maxilliped (M3), first pereopod (P1), second pereopod (P2), and fifth pereopod (P5) (Fig. 1). The largest, most cumbersome appendage is the chelate P2 which was rarely used in grooming (Fig. 3). The smaller, more mobile chelate P1 appendage is better suited to access harder-to-reach and tighter spaces, and it is one of the appendages that are used more frequently in grooming (Fig. 3). The P1-carpal propodal brush also sweeps over the A2 in a quick grooming action, often assisted by the M3. The M3 is frequently used to scrape anterior areas of the body such as other appendages, antennae and antennules; each individual M3 grasps the appendage or antenna/antennule of one side and scrapes from the proximal to the distal end of the groomed appendage. The P5 appendage, or the fifth walking leg, is not chelate like P1 or P2 and was used to scrape posterior parts of the body such as the telson, abdomen or pleopods (Fig. 3). The M3 and P1 were used approximately the same in grooming (Kruskal-Wallis, H=219.88, P<0.001; Mann-Whitney U, z=0.40, P=0.69; Fig. 3) and were the most frequently used grooming appendages (Mann-Whitney U, z=10.24–11.45, P<0.001; Fig. 3). The P2 and P5 appendages were used much less frequently than M3 and P1, but the P5 was used significantly more than the P2 (Mann-Whitney U, z=-4.26, P<0.001; Fig. 3).

Appendages: Morphotypes

Overall, the most frequently used grooming appendages for all individuals (females and the male morphotypes) were the M3 and P1, but there were no significant differences in the frequency of use among the grooming appendages among all three male morphotypes and females. The M3 and P1 were used equally among all groups (Kruskal-Wallis, H=3.55, P<0.001; Fig. 4). The P5 appendage was also used equally between females and the male morphotypes (Mann-Whitney U, z=-0.50, P=0.62; Fig. 4). The P2 appendage was the least used appendage for females and male morphotypes (Fig. 4).

Frequency of Groomed Body Parts: Overall

Grooming of the body parts in terms of frequency were analyzed. The total grooming actions in terms of frequency for all observations (N=94) was 2,838 behaviors. Common grooming behaviors observed include M3 scraping the sensory structures (A1 and A2), P1 brushing the respiratory structures (enclosed gills) and general body grooming

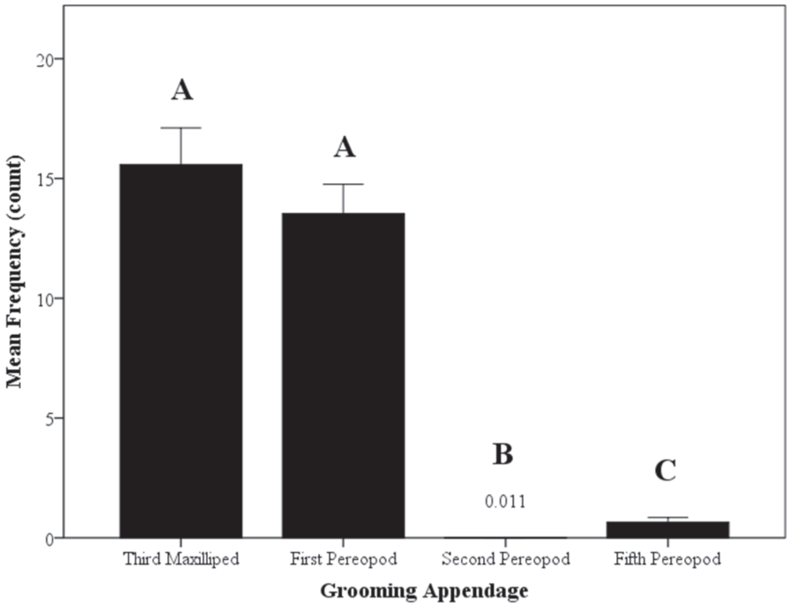

Figure 3. Mean frequency of use of grooming appendages of *Macrobrachium rosenbergii* (N=94) in 30-min time period (mean ± SE). Note: different letters indicate significant differences among use of appendages.

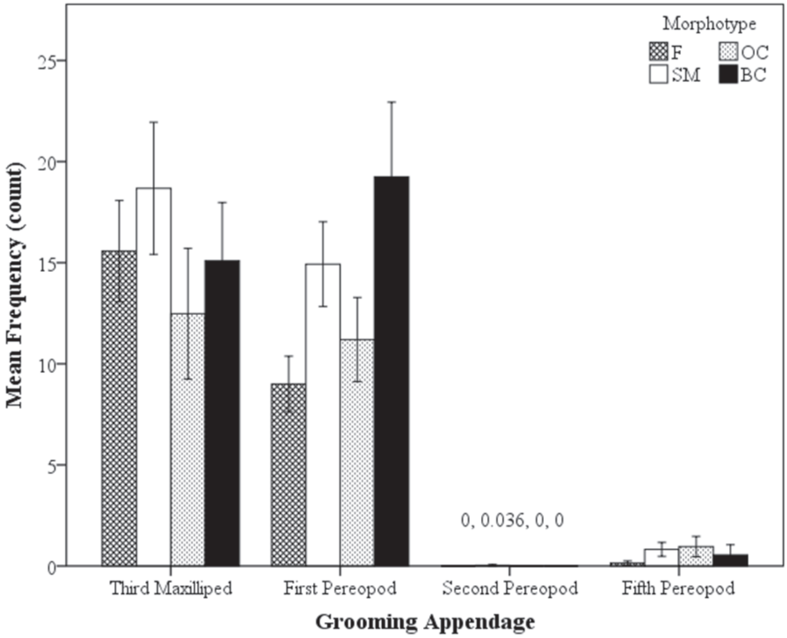

Figure 4. Mean frequency of use of grooming appendages of *Macrobrachium rosenbergii* females (N=21) and male morphotypes (SM: N=28, OC: N=25, BC: N=20) in 30-min time period (mean ± SE). Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males.

by the pereiopods. There was no difference in use of a single appendage between the females and male morphotypes. The most frequently groomed part of the body was the first pereopods (P1) (which are also frequently used grooming appendages) (Fig. 5). As the P1 appendage is a commonly used grooming appendage, it may accumulate fouling material, thus it must be important to keep free of fouling. Although the P1 was the most frequently groomed body part (by the M3 and opposite P1 appendage), there was no significant difference in the frequency of use between the P1 and the next most frequently groomed body area, the antennae (Kruskal-Wallis, H=420.73, P<0.001; Mann-Whitney U, z=-1.83, P=0.067; Fig. 5). The third most frequently groomed area of the body (3rd highest) is the second pereopod (P2) (Fig. 5). The P2 may not be a frequently used as a grooming appendage, but it may be important to keep free of fouling as it is frequently groomed. There was no preference or correlation among the frequencies of grooming parts and location (anterior or posterior body parts) (Mann-Whitney U, z=-0.41, P=0.68; Fig. 5).

Frequency of Groomed Body Parts: Morphotypes

Important sensory, locomotive, and morphological areas of the body were selected among the females and male morphotypes to determine if these areas were groomed equally. These areas and functionality include (1) the walking legs (P5–P8) (locomotive), (2) antennal scale (precision in agonistic interactions and steering and braking function) and (3) pleopods (reproduction and forward swimming). There were no significant differences in the grooming frequency of these selected areas for the females and all male morphotypes (i.e. frequency of grooming antennal scale of females and BC males were equal) (Mann-Whitney U, z=-1.41–0.77, P=0.16–0.97; Fig. 6). There were no significant differences in the mean total frequency of grooming actions for females and male morphotypes (Mann-Whitney U, z=-0.64–0.40, P=0.52–0.85; Fig. 7). All types of individuals had statistically the same number of grooming actions in 30-min trials (Fig. 7).

Time Spent on Body Parts: Overall

Although a part may be frequently groomed, it may not be groomed for a long amount of time. The amount of time spent grooming body parts was analyzed. In all 94 observations, the total time spent observing individuals was 47 hrs. Of those 47 hours, the total time spent grooming by all 94 shrimps was 35,132 sec (9.76 hrs). The part groomed for the longest average time was the gills (Fig. 8), which was not a frequently groomed body part (Fig. 5, 6). The body area groomed for the second highest time was the second pereopods (P2), but there was no significant difference between the time spent grooming these two parts (gills and P2) (Kruskal-Wallis, H=302.66, P<0.001; Mann-Whitney U, z=-0.51, P=0.6067; Fig. 8). There was no obvious correlation or preference in grooming time by location (anterior or posterior body part) (Mann-Whitney U, z=0.96, P=0.34; Fig. 8).

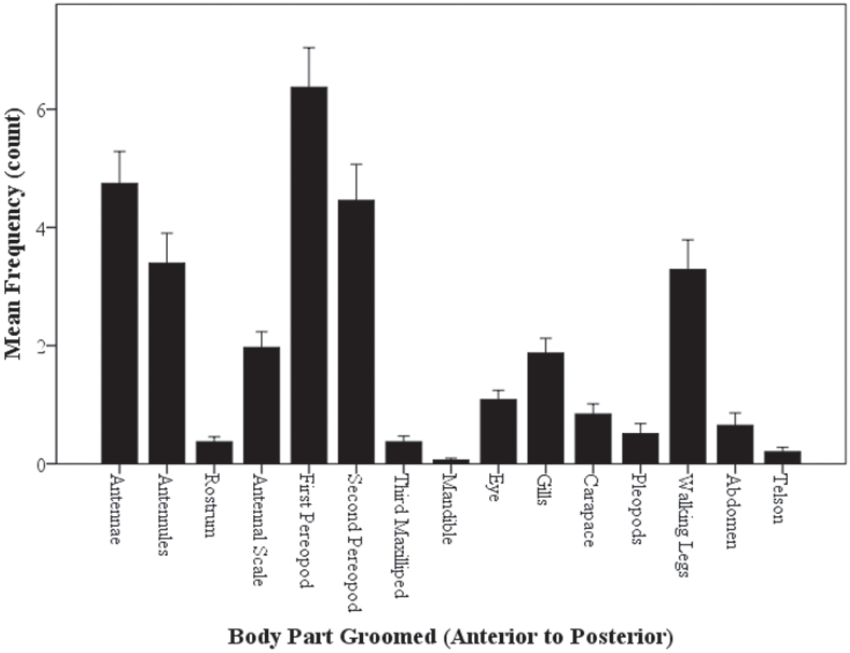

Figure 5. Mean frequency of body parts groomed of *Macrobrachium rosenbergii* (N=94) in a 30-min time period (mean ± SE). Body parts in graph labeled from anterior to posterior, left to right. Note: no significant differences between the two highest body parts, p>0.05.

Time Spent on Body Parts: Morphotypes

When looking at the time spent grooming different body parts in the morphotypes, there were clear differences. There were significant differences in the time spent grooming the second pereopods (P2) between the BC males and all other groups (OC and SM males as well as F (Kruskal-Wallis, H=8.72, P=0.033; Mann-Whitney U, z=-2.73 to -2.02, P=0.006–0.044; Fig. 9). The large BC males spent significantly more time grooming the P2 than all other male morphotypes (Fig. 9). BC males spent the most time grooming most areas of the body (ten out of fifteen body parts; except the antennae, rostrum, mandible, eye and telson) (Fig. 9). In all five cases in which the BC males did not have the highest average time, the SM males spent the most time grooming those areas (Fig. 9). Similarly to the grooming of the body parts in terms of frequency, areas of the body considered important in sensory, locomotive, and morphological functions were selected and analyzed in terms of time: walking legs, the antennal scale and pleopods. There were no significant differences in the time spent grooming these areas among the females and all male morphotypes (i.e. female antennal scale is equal to BC male antennal scale) (Kruskal-Wallis, H=2.00–9.51, P=0.023–0.57; Mann-Whitney U, z=-1.21–0.96, P=0.23–0.92; Fig. 9). There were no significant differences in the mean total time in 30-min trial spent on grooming

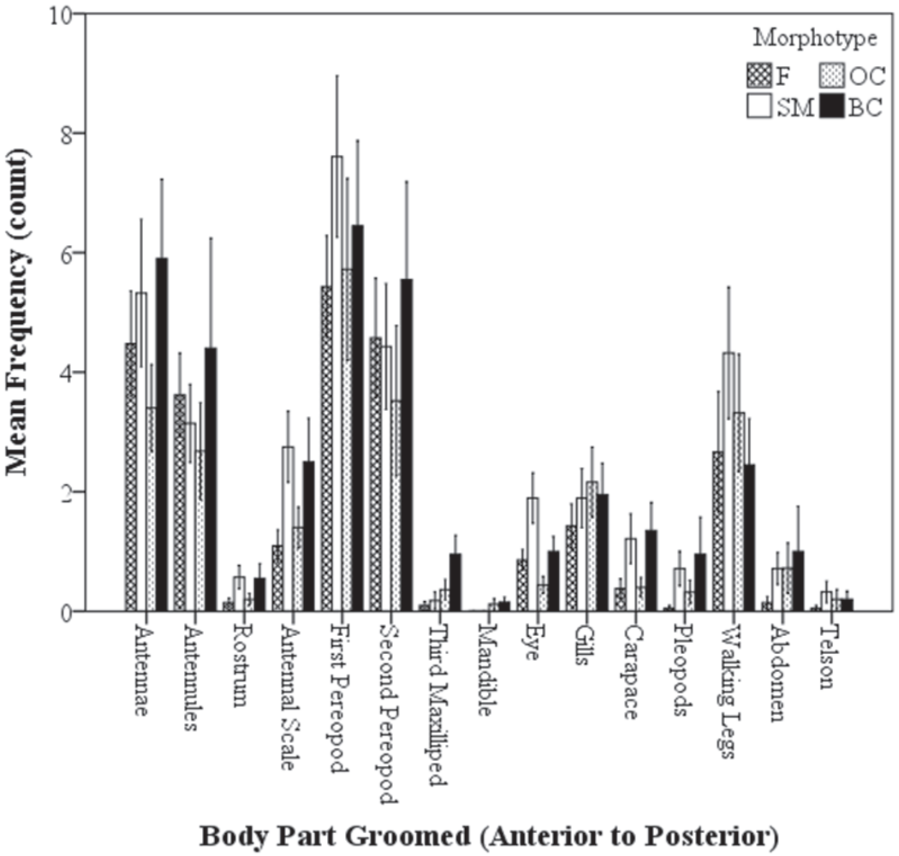

Figure 6. Mean frequency of body parts groomed of *Macrobrachium rosenbergii* females (N=21) and male morphotypes (SM: N=28, OC: N=25, BC: N=20) in a 30-min time period (mean ± SE). Body parts in graph labeled from anterior to posterior, left to right. Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males.

activities for females and male morphotypes (Mann-Whitney U, z=-1.88–1.11, P= 0.06–0.97; Fig. 10). However, there seems to be a trend for BC males to spend more time grooming compared to other male morphotypes and females, but it is not significant; females and male morphotypes spent about the same amount of total time grooming (Fig. 10).

Time Budget

Overall in *Macrobrachium rosenbergii*, a generous proportion of time is spent grooming the body. The average time budget for grooming was 19.3%, indicating up to one-fifth of *Macrobrachium rosenbergii*'s time may be dedicated to grooming when primary behaviors (fighting, mating, etc.) are not present.

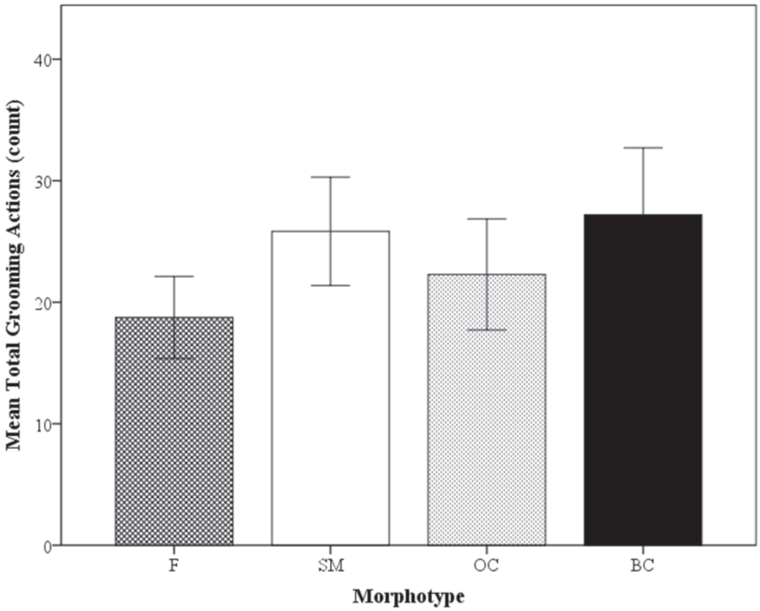

Figure 7. Mean total frequency of grooming behaviors of *Macrobrachium rosenbergii* females (N=21) and male morphotypes (SM: N=28, OC: N=25, BC: N=20) in 30-min time period (mean ± SE). Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males. No significant differences between the groups, p>0.05.

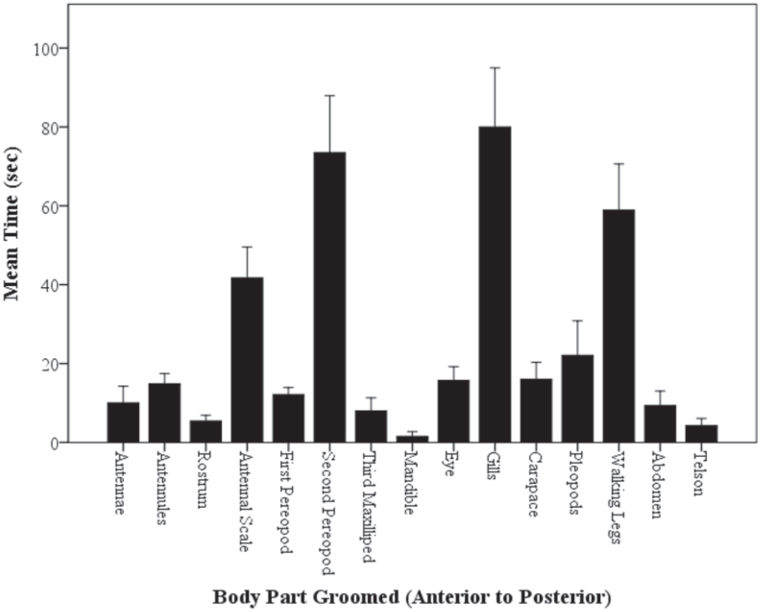

Figure 8. Mean time (sec) of body parts groomed of *Macrobrachium rosenbergii* (N=94) in a 30-min time period (mean ± SE). Body parts in graph labeled from anterior to posterior, left to right. Note: no significant differences between the two highest body parts, p>0.05.

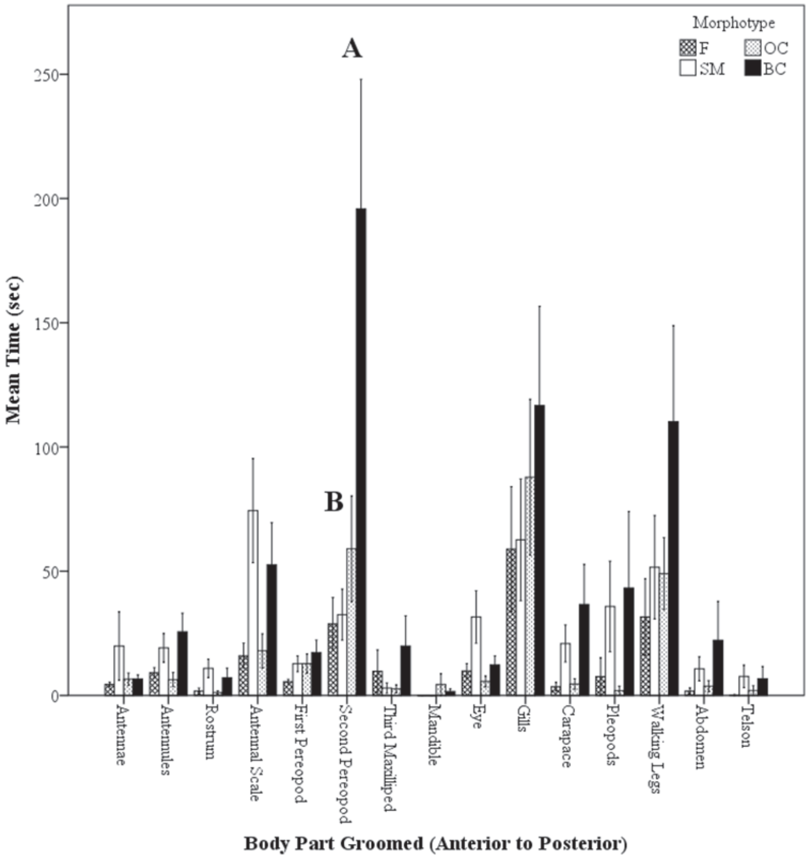

Figure 9. Mean time (sec) of body parts groomed of *Macrobrachium rosenbergii* females (N=21) and male morphotypes (SM: N=28, OC: N=25, BC: N=20) in a 30-min time period (mean ± SE). Body parts in graph labeled from anterior to posterior, left to right. Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males. Different letters indicate significant differences among body parts (B is referring to the females and SM and OC males).

Of the females and male morphotypes, the BC males had the highest average time budget for grooming, 35.2%, which was significantly higher than that of females (10.2%) (Mann-Whitney U, z=-2.93, P=0.0033; Fig. 11) and OC males (13.6%) (Mann-Whitney U, z=2.69, P=0.0072; Fig. 11). The grooming time budget for females, SM males and OC males were 10.2%, 19.8% and 13.6%, respectively (Fig. 11).

Social Grooming Observations (Observations #2)

In all social observations (N=8), not one grooming behavior occurred. Hence, the observations were ended prematurely at a lower sample size compared to the other observations (Observations #1 and #3). The shrimps were in a social situation where many behaviors such as searching for mates, displaying, touching and grooming can occur. The grooming time budget was 0% for all observations.

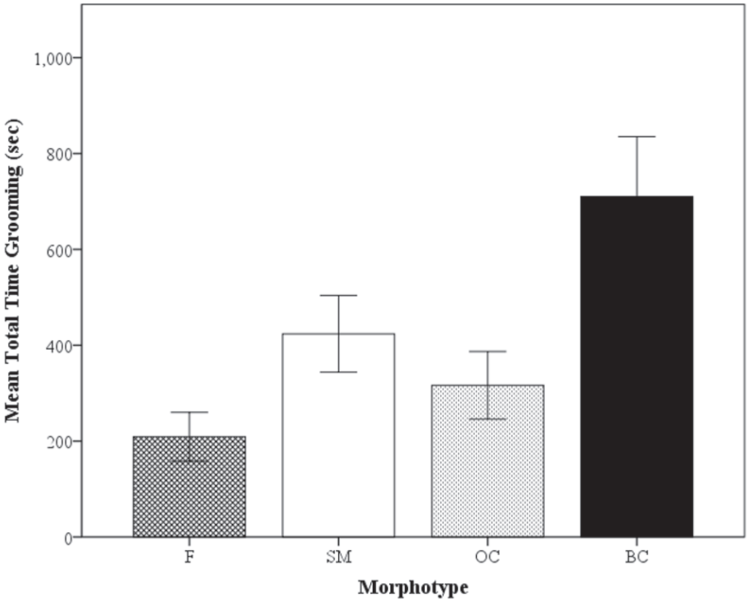

Figure 10. Mean total time (sec) spent grooming of *Macrobrachium rosenbergii* females (N=21) and male morphotypes (SM: N=28, OC: N=25, BC: N=20) in 30-min time period (mean ± SE). Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males. No significant differences between the groups, p>0.05.

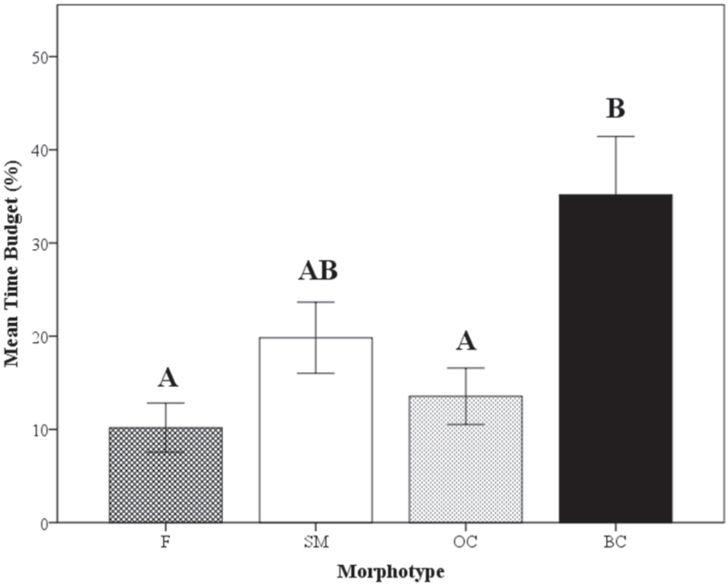

Figure 11. Mean time budget for grooming of *Macrobrachium rosenbergii* morphotypes (F: N=21, SM: N=28, OC: N=25, BC: N=20) in 30-min time period. Overall mean time budget of species is 19.3%. Note: BC = blue-clawed males; F = females, OC = orange-clawed males; SM = small males. Different letters indicate significant differences among groups.

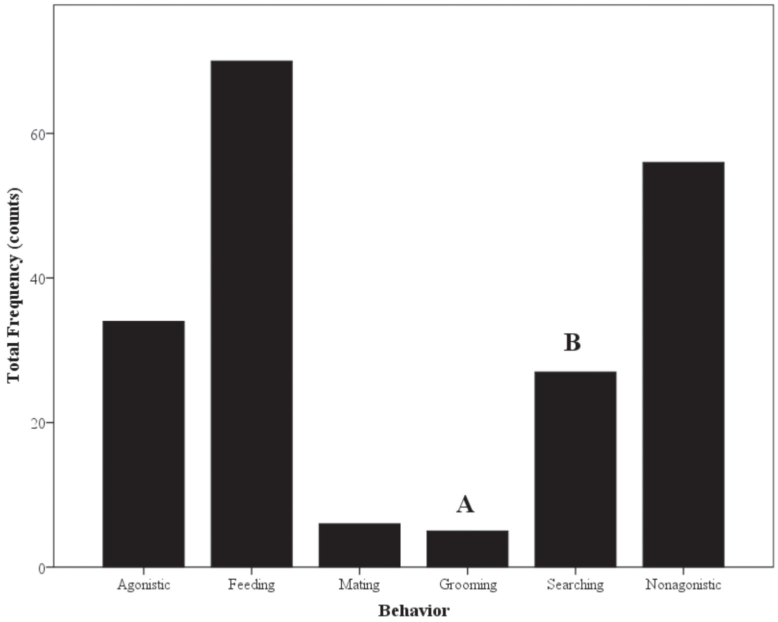

Figure 12. Mean frequency of behaviors during agonistic observations (N=20) of *Macrobrachium rosenbergii*. Note: Different letters indicate significant differences among morphotypes. Note: different letters indicate significant differences among behaviors.

Agonistic Grooming Observations (Observations #3)

The paired shrimps (N=20; Table 1) physically interacted often (Fig. 12). No social grooming (allogrooms) occurred in any trial. The most frequent behavior was feeding and the least common behavior was grooming, making up 35% and 2.5% of the grooming activites, respectively (Fig. 12). While there was no food given, shrimps picked up particulate matter in the water column that had settled on the bottom. Some examples of non-agonistic interactions that occurred include cheliped or antennae touching and antennal flicking. There was a significant difference in the frequency of these behaviors, with grooming occurring significantly less than all other behavioral categories except mating (Mann-Whitney U; z=-3.52; P<0.001; Chi-squared test; χ^2=104.5; P<0.001; Fig. 12). Mating was not compared to grooming frequencies due to mating only possible in a fraction of the treatments where females and males were present (Treatments 4, 7 and 9; Table 1).

The behavior that occurred for the longest time was non-agonistic interactions and grooming occurred for the shortest amount of time (Fig. 13). There were significant differences in the time spent among the various behaviors with grooming lasting significantly less time than all other behaviors except mating (Mann-Whitney U; z=-4.00; P<0.001; Chi-squared test; χ^2=13,610.7; P<0.001; Fig. 13). The grooming time budget was 0.31% in these treatments (N=20).

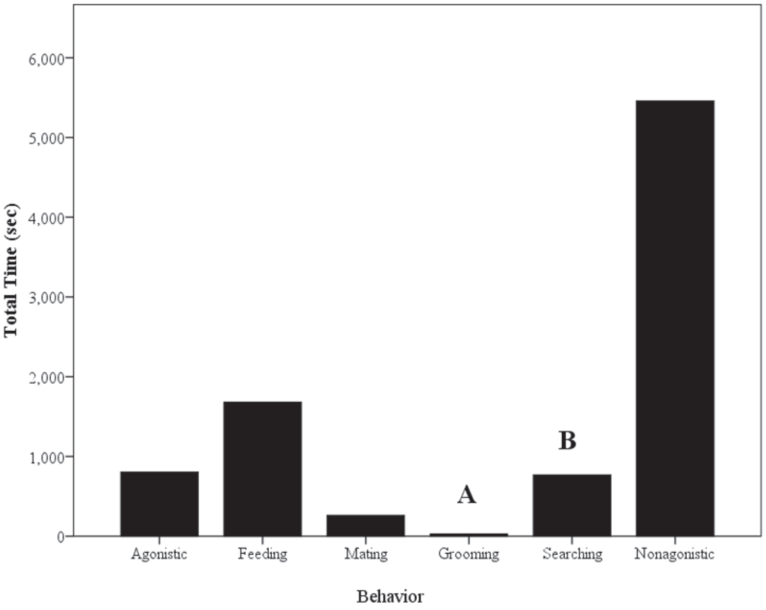

Figure 13. Mean time (sec) spent on behaviors during agonistic observations (N=20) of *Macrobrachium rosenbergii*. Note: different letters indicate significant differences among behaviors.

Discussion

Grooming in *Macrobrachium rosenbergii*

Overall, *Macrobrachium rosenbergii* showed similar grooming behaviors compared to other caridean shrimps including the usage of specific grooming appendages (third maxillipeds, first, second and fifth pereopods) (Bauer 1978, 1981, Felgenhauer and Schram 1979), priority of body parts groomed (ie: P1 groom the gills for respiration, M3 scrape the antennules for sensory reception) (Bauer 1977, 1979) and time budget dedicated to grooming activities (Table 2). Each day shrimps spend a large portion of their time grooming, leaving fewer hours to accomplish other activities such as finding suitable habitat, maintaining and defending that habitat, resting, reproduction, and searching for food. The amount of time caridean shrimps spend grooming suggests there must be an important benefit for the activity. Bauer (1979, 1989, 2004) has shown that grooming is an adaptive behavior especially in caridean crustaceans due to the development of complex structures and behaviors related to grooming.

Although there were similarities in the grooming behaviors of *Macrobrachium rosenbergii* and other carideans, the male morphotypes of *M. rosenbergii* differed in grooming behaviors. These morphotypes are known to differ in their behavior based on their niche in the social hierarchy, and therefore it is plausible their grooming behaviors and time dedicated to grooming may differ (Ra'Anan and Sagi 1985). BC males spent a significantly longer time grooming the second pereopods compared to

Table 2. Grooming time budgets of caridean shrimps.

Species	Grooming Time Budget (%)	Presence of Conspecifics?	Reference
Heptacarpus pictus	27%	No	Bauer 1977
Macrobrachium grandimanus	25%	No	VanMaurik and Wortham 2011
Macrobrachium rosenbergii	19%	No	Current study (Observations #1)
Macrobrachium rosenbergii	0%	Partial	Current study (Observations #2)
Macrobrachium rosenbergii	0.31%	Yes	Current study (Observations #3)

all other males and females, indicating this area may be important for this hierarchical group. BC males are the terminal molt stage and are not able to rid of fouling by molting (Amir Sagi, personal communication). The second pereopods of BC males are used to dominate other males, as well as for protection and defense of females. As a result, BC males seem to invest much time and energy to remove fouling.

The SM and BC male morphotypes differ in their behavior yet they have the highest total frequency and time of grooming actions along with the highest time budgets for grooming. This may be attributed to the relative efficiencies of their grooming activities: SM males are highly mobile and may experience greater fouling pressures (Ra'Anan and Sagi 1985, Bauer 1989) and an increased grooming need, resulting in much time and effort dedicated to the removal of fouling agents. BC males have lower fouling pressures due to their limited mobility but higher pressures as they are unable to molt and rid of fouling compared to SM males. Grooming in BC males may not be very efficient due to cumbersome grooming appendages, as grooming frequently occurs (Ra'Anan and Sagi 1985). It is also likely that BC are equally efficient at grooming but have a higher need for grooming since they are the terminal molt stage.

Although BC males have the highest grooming time budget, it appears that most of this time is spent in the grooming of the P2 appendage. The BC males groomed the P2 appendage frequently and for a long time, which may be due to the setal patch located on the propodus. The setal patch may participate in displays to ward off other males from territories (Correa et al. 2000). The P2 appendage is also used in the protection and defense of females. The fact that the BC males dedicate much time to the grooming of this appendage indicates it is may be important in maintaining the dominance position of these males in the social hierarchy.

We found that primary (higher priority) behaviors such as feeding and defense are of greater importance and should occur more frequently than grooming when primary behaviors are possible. Grooming was absent or rare when primary actions occurred (social grooming observations, Observations #2), therefore grooming should be considered as a secondary behavior, as hypothesized by Bauer (1989). Females with embryos were not used in grooming observations; the time spent on pleopod grooming would likely be higher if they were included (Bauer 1979). When individuals were in contact with other conspecifics, primary actions such as fighting, feeding, mating, searching and non-agonistic interactions occurred more often and longer, taking precedence over grooming (Table 2). Although grooming is likely of lower priority than primary actions, it is still an important adaptive behavior to fouling pressures and it maintains vital, primary functions such as locomotion, respiration, chemosensory reception and reproduction.

Conclusions

As hypothesized, *Macrobrachium rosenbergii* showed similar grooming behaviors and grooming time budgets compared to other caridean shrimps indicating grooming behaviors have evolved in response to the fouling pressures experienced in an aquatic environment. We found that the BC males dedicate much time to the care of the P2 appendages which are used in displays, protection and defense. This behavior contributes to the high time budget for grooming in the BC males. Grooming was also hypothesized to be a secondary behavior, only occurring when other behaviors are not essential (Bauer 1989). The results of this study indicate grooming in shrimps is a secondary behavior as demonstrated by the behaviors of the commercially important shrimp, *Macrobrachium rosenbergii*.

Macrobrachium rosenbergii is frequently grown in aquaculture (New and Nair 2012) with the BC males being the largest in size and most valuable in terms of yield and profit, even though all individuals (SM, OC, BC and females) are grown and may be sold for consumption (New and Valenti 2000, FAO 2004). Once these shrimps reach a certain age or size, they are usually exported to an outdoor grow-out tank, which is subject to agricultural or industrial runoff, sedimentation, algal blooms and fouling organisms such as bacteria or invertebrates (New and Valenti 2000, Bauer 2002). As a result, fouling is very likely high, leading to survival implications (decreased respiration, movement, sensory reception or defense) during the intermolt period. The best economic interest of aquaculture farmers should be to have the lowest mortality rate of shrimps in order to allow them to grow to the largest, most profitable size since all BC males must survive past the SM and OC male phase to develop until the most profitable BC male. Besides water quality and filtration, the population density in aquaculture tanks is an important factor regulating the behaviors of shrimps (high densities correlate with greater occurrence of primary behaviors such as mating and defense). Therefore, yields depend on the size of the hierarchial groups in the grow-out tanks or ponds. Grooming behaviors are important to understand as antifouling adaptation, particularly in high-density populations such as aquaculture settings.

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RESEARCH ARTICLE

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Molecular and morphological differentiation between two Miocene-divergent lineages of Amazonian shrimps, with the description of a new species (Decapoda, Palaemonidae, *Palaemon*)

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Abstract

Palaemon carteri (Gordon, 1935) and *P. ivonicus* (Holthuis, 1950) are morphologically similar species of South American freshwater shrimps. Past studies have questioned the taxonomic status of both species, which are supposed to have partially sympatric geographic distributions in the Amazon basin. We analyzed a 550 bp fragment of the mitochondrial 16S rRNA gene from these Amazonian *Palaemon* species as well as from 11 palaemonids as the outgroup. Additionally, we checked diagnostic characters of the genus and family as well as other morphological characters that have been little explored before. *Palaemon carteri* and *P. ivonicus* are allocated in two sister lineages, with wide genetic divergence and little morphological differentiation. The divergence time between these lineages was estimated as approximately 10 million years ago. Both molecular and morphological data support the taxonomic validity of both *Palaemon carteri* and *P. ivonicus*, refuting the hypothesis of synonymy. In addition, a new species, *Palaemon yuna* **sp. n.**, closely related to *P. ivonicus*, is described. Our findings indicate that these species can be differentiated using the projection of the anterolateral margin and anterolateral spine of the first antennular segment, shape of the rostrum, and relative size of the appendix masculina.

Keywords
Amazon basin, divergence time, freshwater shrimp, *Palaemon yuna*, taxonomy

Introduction

The genus *Palaemon* Weber, 1795 comprises 84 marine, estuarine and freshwater species in tropical and subtropical regions, including *Palaemonetes* Heller, 1869, which was recently considered to be a junior synonym of *Palaemon* by De Grave and Ashelby (2013), and the new species described here. Four strictly freshwater species occur in South America, distributed in the Amazon, Orinoco and Paraguay/lower Paraná River basins: *Palaemon carteri* (Gordon, 1935), *P. ivonicus* (Holthuis, 1950) *P. mercedae* (Pereira, 1986) and a new species described herein.

Palaemon carteri and *P. ivonicus* are freshwater species with abbreviated larval development (see Magalhães 1986 for larval description of *P. ivonicus*) and are supposed to occur sympatrically in the Amazon basin as well as being very similar morphologically. They have been distinguished primarily based on rostral characters and on the position of the branchiostegal tooth. Holthuis (1952) stated that *Palaemon ivonicus* has the lower margin of the rostrum with two or three teeth, the rostrum rather high and straight, and the branchiostegal tooth removed a considerable distance from the anterior margin of the carapace, with its tip failing to reach beyond this margin. On the other hand, *P. carteri* has the lower margin of the rostrum with four to seven teeth; the rostrum slender, often curved upwards; and the branchiostegal tooth removed a short distance from the anterior margin of the carapace. Nevertheless, some studies have shown that the main characters currently used (position of the branchiostegal tooth, number of rostral teeth, and rostral shape) are not enough to differentiate *P. ivonicus* from *P. carteri* consistently (Gomes-Corrêa 1977, Odinetz-Collart and Enriconi 1993, García-Dávila and Magalhães 2003). Therefore, the high interspecific morphological similarity and intraspecific variability found in these species raised doubts as to whether these two nominal species represent distinct biological entities (Gomes-Corrêa 1977, Odinetz-Collart and Enriconi 1993, García-Dávila and Magalhães 2003, García-Dávila et al. 2005).

A multivariate morphometric approach was applied in order to confirm whether the two species constitute separate biological entities in the Amazon basin, and a wide plasticity and overlap in these characters among populations was found (García-Dávila et al. 2005), enhancing the hypothesis of synonymy (Gomes-Corrêa 1977). However, populations from black- and clear-water river systems (Negro and Tapajós river basins, respectively) formed a group slightly distinct from those from white-water river systems (Solimões/Amazon river basin), based on morphometric patterns (García-Dávila et al. 2005; see Sioli 1984 for a characterization of the three Amazonian hydrological systems).

The occurrence of *P. ivonicus* in the Amazon, Orinoco and Paraguay/lower Paraná basins, however, might indicate a Miocene origin for these lineages. In this period the Orinoco and Amazon basin were widely connected and different sequences of capture of headwater might have resulted in dispersal of species across boundaries of the Amazon and Paraguay basins during the Tertiary (see Lundberg et al. 1998 and Magalhães et al. 2005), which means that these populations could have had enough time to undergo speciation.

Regarding the morphological variability of this group, our study aimed to test the taxonomic status of *P. ivonicus* and *P. carteri*, as well as to verify the presence of a new species from the Negro River basin, on the basis of partial sequences of the large ribosomal subunit 16S and morphological analyses.

Methods

Abbreviations and symbols used

CCDB	Crustacean Collection of the Biology Department, Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil.
CL	Carapace length (measured from the posterior margin of the orbit to the posterior margin of the carapace).
CNCR	National Crustacean Collection of the Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico.
INPA	Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil.
OUMNH-ZC	Zoological Collections of the Oxford University Museum of Natural History, Oxford, England.
MNRJ	National Museum of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
MPEG	Museu Paraense Emilio Goeldi, Belém, Brazil.
MV	Museum of Victoria, Melbourne, Australia.
MZUSP	Museum of Zoology of the University of São Paulo, São Paulo, Brazil.
NHM	Natural History Museum, London, England.
USNM	National Museum of Natural History (United States National Museum), Smithsonian Institution, Washington, D.C., USA.
MZUCR	Universidad de Costa Rica, Museo de Zoología, San José, Costa Rica.

♂: male, ♀: female, ♀ov: ovigerous female.

Sampling

Specimens from several localities were obtained from field collections as well as from visits to and loans from the above-mentioned carcinological collections (Fig. 1). Collected specimens were euthanized on ice, transferred to 96% ethanol, morphotyped under a Leica M205C stereomicroscope, and finally preserved in 80% ethanol for deposit in

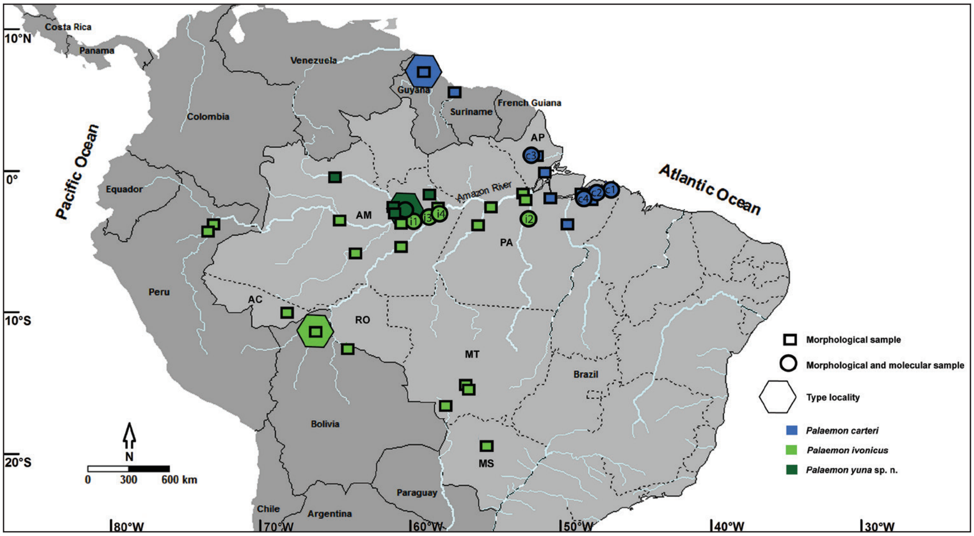

Figure 1. Sample sites of *Palaemon carteri*, *P. ivonicus* and *P. yuna* sp. n. c1–Bragança, Pará; c2–Santa Maria do Pará, Pará; c3–National Forest of Amapá, Amapá; c4–Belém, Pará; i1–Solimões River, near Manaus, Amazonas; i2–Xingu River, Altamira, Pará; i3 and i4–Itacoatiara, Amazonas; AC-Acre; AM-Amazonas; AP-Amapá; MS-Mato Grosso do Sul; MT-Mato Grosso; PA-Pará and RO-Rondônia.

the CCDB. Specimens from donations and loans were also assigned to morphotypes, and each morphotype was deposited in the CCDB or in the original collection. The collections of species conducted in this study complied with current applicable state and federal laws of Brazil [FLC's authorization from ICMBio (No. 25329); DIFAP/IBAMA/126/05; and permanent license to FLM for collection of Zoological Material No. 11777-1 MMA/IBAMA].

Molecular data

DNA extraction, amplification and sequencing protocols followed Schubart et al. (2000) with modifications as in Mantelatto et al. (2007, 2009) and Pileggi and Mantelatto (2010). Total genomic DNA was extracted from the muscle tissue of the abdomen. An approximately 550 bp region of the mitochondrial 16S rRNA gene was amplified from four specimens of *P. carteri*, four of *P. ivonicus*, one of *P. yuna* sp. n. and ten of other palaemonids (Table 1). The amplification was performed by polymerase chain reaction (PCR) in an Applied Biosystems Veriti 96 Well Thermal Cycler® (thermal cycles: initial denaturing for 5 min at 95 °C; pairing for 40 cycles: 45 s at 95 °C, 45 s at 52 °C, 1 min at 72 °C; final extension 5 min at 72 °C) with universal 16S mtDNA primers 1472 (5'-AGATAGAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996) and 16S-L2 (5'-TGCCTGTTTATCAAAAACAT-3') (Schubart et al. 2002). PCR products were purified using Sure Clean (Bioline) and sequenced with the ABI Big Dye® Terminator Mix (Applied Biosystems, Carlsbad, CA) in an ABI 3730 XL DNA Analyzer (Applied

Table 1. Specimens of Palaemonidae used for the phylogenetic analyses. CCDB: Crustacean Collection of the Department of Biology of the Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, University of São Paulo; CNCR: National Crustacean Collection of the Instituto de Biología, Universidad Nacional Autónoma de México; MPEG: Museu Paraense Emilio Goeldi; MV: Museum Victoria; MZUCR: Museo de Zoología, Universidad de Costa Rica.

Taxon	Locality	Collection accession number	GenBank
Creaseria morleyi (Creaser, 1936)	Yucatan Peninsula, Mexico	---	EU448997
Palaemon carteri (Gordon, 1935)	(c1) Jequiri, Bragança, Pará, Brazil	MPEG 0787	KF923721
Palaemon carteri (Gordon, 1935)	(c2) Santa Maria do Pará, Pará, Brazil	CCDB 4339	KF923720
Palaemon carteri (Gordon, 1935)	(c3) Japim stream, National Forest of Amapá, Amapá, Brazil	MPEG 1108	KF923727
Palaemon carteri (Gordon, 1935)	(c4) Mocambo, Belém, Pará, Brazil	MPEG 0628	KF923730
Palaemon gracilis (Smith, 1871)	Pacific coast, Costa Rica	CCDB 3402	KF923714
Palaemon hancocki Holthuis, 1950	Golfo Dulce, Puntarenas, Pacific coast, Costa Rica	MZUCR 2477-02	KF923715
Palaemon intermedius (Stimpson, 1860)	Victoria, Australia	MV J60843	KF923725
Palaemon ivonicus (Holthuis, 1950)	(i1) Solimões River, Manaus, Amazonas, Brazil	CCDB 1435	KF923717
Palaemon ivonicus (Holthuis, 1950)	(i2) Xingu River, Altamira, Pará, Brazil	MPEG 0715	KF923726
Palaemon ivonicus (Holthuis, 1950)	(i3) Poranga, Itacoatiara, Amazonas, Brazil	CCDB 4632	KF923728
Palaemon ivonicus (Holthuis, 1950)	(i4) Itacoatiara, Amazonas, Brazil	CCDB 4716	KF923729
Palaemon kadiakensis (Rathbun, 1902)	Convent, Louisiana, USA	CCDB 1600	KF923718
Palaemon longirostris H. Milne Edwards, 1837	Guadiana River, Portugal	CCDB 2750	KF923724
Palaemon northropi (Rankin, 1898)	Mamanguape River, Paraíba, Brazil	CCDB 4332	KF923722
Palaemon pandaliformis (Stimpson, 1871)	Ilha Comprida, São Paulo, Brazil	CCDB 813	KF923713
Palaemon pugio (Holthuis, 1949)	River Delta, Gautier, Mississippi, USA	CCDB 3804	KF923723
Palaemon ritteri Holmes, 1895	Bahía Wafer, Puntarenas, Pacific coast, Costa Rica	MZUCR 2396-04	KF923719
Palaemon suttkusi (Smalley, 1964)	Salado River, Zaragoza, Mexico	CNCR 25864	KF923712
Palaemon yuna sp. n.	Lago Tupé beach, Negro River, Manaus, Amazonas, Brazil	CCDB 4866	KF923716

Biosystems, Foster City, CA) following Applied Biosystems protocols. All sequences were confirmed by sequencing both strands. A consensus sequence for the two strands was obtained using the BIOEDIT software (version 7.0.5) (Hall 2005).

Phylogenetic analysis

Sequence alignments were conducted in MAFFT alignment software (version 7.058) with default settings (Katoh and Standley 2013). Estimates of uncorrected genetic divergence (p-distance) for sequence pairs were conducted in MEGA (version 5.2.2) (Tamura et al. 2011), and statistical selection of models of nucleotide substitution in jModelTest (version 2.1.4) (Darriba et al. 2012). The gene dataset was tested for nucleotide substitution saturation using the test by Xia et al. (2003) implemented in DAMBE v. 5.3.48, which revealed no significant saturation ($P < 0.05$) for symmetrical trees.

We performed four phylogenetic reconstructions: two by Bayesian inference, one by maximum likelihood and one by parsimony. All analyses were conducted using *Creaseria morleyi* (Creaser, 1936) as the outgroup. A consensus tree of the two Bayesian and maximum likelihood analyses was constructed by 50% majority-rule in the Mesquite package (2.75, build 566). Only posterior probabilities and bootstrap values above 50% are shown. All other software settings not mentioned below were maintained as default.

The Bayesian inferences were performed in the MrBayes software (version 3.2.2) (Ronquist et al. 2012). The Metropolis-coupled Markov chain Monte Carlo was used to empirically determine the posterior probability distribution of trees, branch lengths and substitution parameters. The nucleotide substitution model assumed was the 4by4 with general time-reversibility, gamma-distributed rate variation across sites and invariable sites (GTR+Γ+I). Five gamma rate categories were used. The prior probability distributions on the parameters of the model were maintained as default. Aiming to evaluate possible effects of overfitting, we also performed a second Bayesian inference using a simpler model (Hasegawa-Kishino-Yano with gamma-distributed rates: HKY+Γ) indicated by the Bayesian information criterion. In this second analysis, the parameter values given by jModelTest (nucleotide frequencies, transition/transversion ratio, shape of the gamma distribution and proportion of variable sites) were used to define the prior probability distributions on the parameters of the model. Both analyses were carried out with 10^7 generations in two independent runs, with one cold and four heated parallel chains. The parameter values were saved once every 1,000 rounds. The runs were stopped if they had reached stationarity (average standard deviation of the split frequencies below 0.01). The first quarter of parameters and trees was discarded (burn-in of 25%); see Ronquist et al. (2009) for further details.

The maximum likelihood (ML) analysis was conducted in the RAxML program (7.6.3) (Stamatakis 2006) implemented in CIPRES ("Cyberinfrastructure for Phylogenetic Research"; http://www.phylo.org). The consistency of topologies was measured by the bootstrap method. The number of bootstrap replicates (650) as well as the proportion of invariable sites was defined by RAxML.

The maximum parsimony analysis was performed using the branch-and-bound algorithm in PAUP (version 4.0 for Unix/Linux). We conducted a bootstrap analysis with 1,000 replicates. Gaps, missing and ambiguous characters were excluded in this analysis.

Molecular dating

The likelihood ratio test (LRT) was used to test the null hypothesis that the data evolved under a molecular clock. The likelihoods for the molecular trees, under the GTR+Γ+I model, with and without an imposed molecular clock were compared using the LRT calculator in jModelTest. The assumption of a molecular clock could not be rejected ($P = 0.51$). We used both a strict molecular clock and a relaxed molecular clock with independent gamma rates (IGR) in MrBayes, under the settings mentioned above, in order to estimate an approximate divergence time between the *P. carteri* and *P. ivonicus* / *P. yuna* lineages. We assumed the rate of 0.0083 substitutions per site per million years to corrected divergence values for the 16S rRNA gene. This rate was estimated for neotropical palaemonid species of *Palaemon*, assuming that the isolation of the transisthmian estuarine sibling species *P. ritteri* Holmes, 1895 and *P. paivai* Fausto Filho, 1967 (CCDB 4334) occurred about three million years ago (Carvalho et al. unpublished data), as demonstrated for other estuarine carideans (Knowlton and Weigt 1998, Hurt et al. 2009). This rate is also compatible with rates estimated for other decapods (0.006–0.009) by Schubart et al. (2000) for the same gene.

Morphological analysis

Once the phylogenetic relationships based on molecular data were known, we analyzed adult morphological characters of South American species of *Palaemon* in order to verify the morphological support for the clades obtained, as well as provide new diagnoses for the *P. carteri* / *P. ivonicus* / *P. yuna* group.

We checked out the diagnostic characters that were traditionally used to differentiate *P. carteri* from *P. ivonicus*: position of the branchiostegal tooth, number of rostral teeth, and rostral shape (Holthuis 1952, Melo 2003). We also analyzed other characters that were traditionally used in Palaemonidae, as well as others that have been little explored in the taxonomy of the group, such as the projection of the anterolateral margin of the first antennular segment, the anterolateral spine of the first antennular segment, and the relative size of the appendix masculina.

The search for morphological differences among species was conducted using the optimized comparison method described below. Initially, all pairwise differences found between the specimens from each clade in the tree were listed. Those differences with more than 80% constancy were chosen for further validation, using the remaining specimens from the same lots used in the molecular analyses. The consistency of each difference was evaluated step-by-step, analyzing blocks of 10 specimens from each clade. Characters with less than 80% constancy were discarded and no longer analyzed in the following blocks. Additional blocks of specimens from lots that were not included in the molecular analyses were used for a final check.

The diagnostic characters found were verified in the original descriptions (Gordon 1935, Holthuis 1950a, 1952) as well as in the type series of *P. carteri* and *P. ivonicus* in order to confirm the taxonomic entity of each clade.

Results

The two Bayesian analyses, maximum likelihood and parsimony analyses indicated the same topology. The mean standard deviation of the split frequencies after 10^7 generations was less than 0.003 for both GTR+Γ+I and HKY+Γ models. No important differences were found between the two Bayesian inferences using each model, since only slight differences were noted in the posterior probabilities of the clades and branch lengths, indicating that there is no perceptible effect of model overfitting. Similarly, only dissimilarities in the values of node support were observed between the maximum likelihood and the Bayesian inferences, with the GTR+Γ+I implemented in both analyses.

Of the 435 aligned positions (after exclusion of gaps, missing and ambiguous characters), 193 sites were variable, of which 54 (28%) were parsimony-informative. The parsimony reconstruction also showed more than 50% bootstrap values for all clades indicated by the previous analyses, with high support for most of them.

The monophyly of the *P. carteri* / *P. ivonicus* / *P. yuna* group was indicated in all analyses, although this clade had weak bootstrap support in the parsimony and maximum-likelihood methods. All analyses supported the existence of two sister lineages for the specimens of this group (Fig. 2). The "Carteri" lineage allocated specimens from eastern Amazonia, assigned to *P. carteri*. The "Ivonicus/yuna" lineage comprised specimens from central and western Amazonia, which were assigned to *P. ivonicus* and *P. yuna* sp. n. The population from the black water Negro River, assigned to *P. yuna* sp. n. (Fig. 3), clearly fell outside the clade that comprised specimens of *P. ivonicus* from other regions of the Amazon basins (with white and clear water).

The wide genetic dissimilarity between the "Carteri" and "Ivonicus/yuna" lineages (from 10.6% to 13.7%), compared among the representative members of the genus used here (from 3.5% to 23.0%; data not shown), also supports the hypothesis that *P. carteri* and *P. ivonicus* are valid species, as well as clearly genetically divergent (Fig. 4). The intralineage genetic variability was 0–3.6% in "Carteri" and 0–4.3% in "Ivonicus/yuna". The interspecific dissimilarity between *P. ivonicus* and *P. yuna* was 4.1–4.3%.

The divergence time between the "Carteri" and "Ivonicus/yuna" lineages, based on the 16S rRNA gene, was estimated as approximately 10 million years ago. This mean value varied between ~9 and ~11 Ma in our analysis, depending on the molecular-clock model used, as well as other parameter settings such as node constraints. The 95% credible intervals (highest posterior density–HDP) were 5.5–14.3 Ma using a relaxed clock, and 8.2–14.0 Ma using a strict clock.

Morphological characters supported these lineages. The morphological analysis (including the type series of all three species) of 122 specimens of *P. carteri*, 333 specimens of *P. ivonicus* and 125 specimens of *P. yuna* sp. n. indicated that the projection of the anterolateral margin and the anterolateral spine of the first antennular segment were useful characters to differentiate these lineages, as long as adult specimens were considered. The "Carteri" lineage had the projection of the anterolateral margin of the first antennular segment slightly shorter, not reaching the dorsal distal margin of the second segment. Additionally, the anterolateral spine of the first antennular segment usually reached the middle of the projection of the anterolateral margin (Fig. 5a). On the other hand, the "Ivonicus/yuna" lineage had the projection of the anterolateral margin of the first antennular segment longer, reaching the dorsal distal margin of the

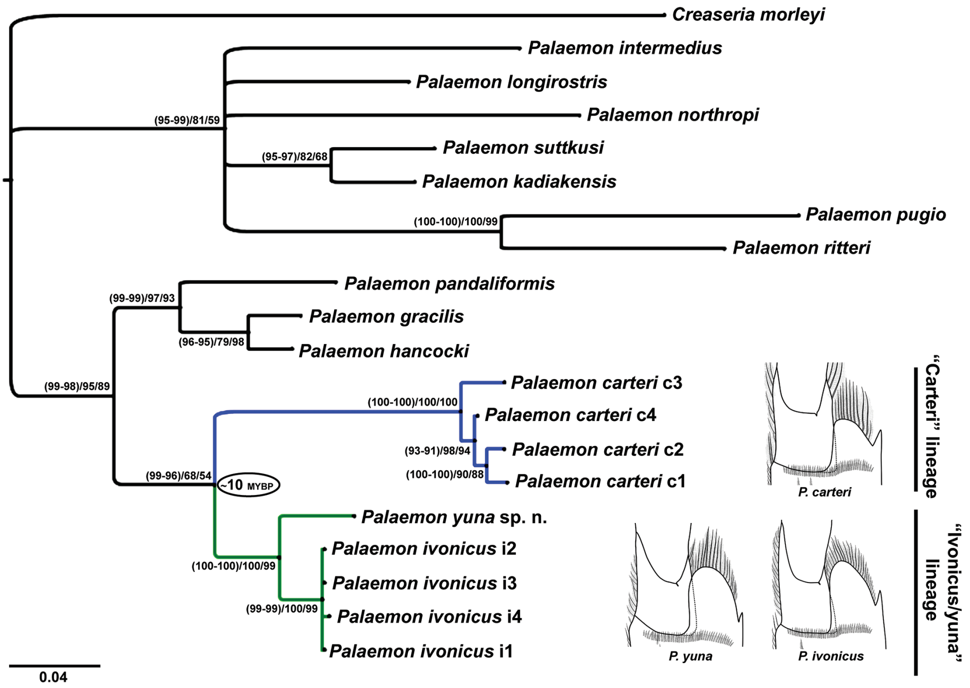

Figure 2. Bayesian (GTR+Γ+I and HKY+Γ models) and maximum likelihood 50% majority-rule consensus tree. Numbers in the nodes represent posterior probabilities (GTR+Γ+I and HKY+Γ, respectively), and bootstrap value for maximum likelihood and parsimony analyses, respectively. c1–Bragança, Pará; c2–Santa Maria do Pará, Pará; c3–National Forest of Amapá, Amapá; c4–Belém, Pará; i1–Solimões River, near Manaus, Amazonas; i2–Xingu River, Altamira, Pará; i3 and i4–Itacoatiara, Amazonas. MYBP–million years before present.

Figure 3. *Palaemon yuna* sp. n. Holotype, male, CCDB 4865, habitus, lateral view.

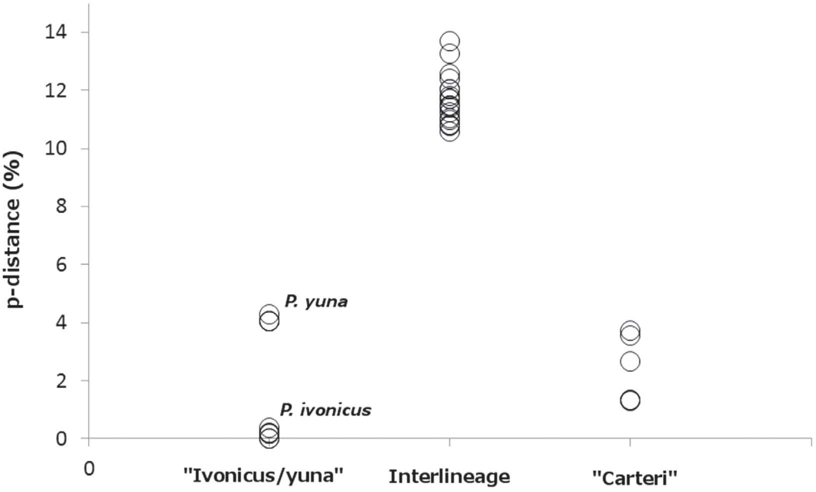

Figure 4. Intralineage and interlineage uncorrected genetic distance values for the "Ivonicus/yuna" and "Carteri" lineages.

second segment. Moreover, the anterolateral spine of the first antennular segment did not reach the middle of the projection of the anterolateral margin (Fig. 5b–c).

The rostral characters (shape, relative size and number of ventral teeth of the rostrum) were helpful to differentiate *P. ivonicus* from *P. yuna* sp. n. (Fig. 6). *Palaemon ivonicus* had the rostrum high, straight or slightly curved upward, not overreaching the scaphocerite; the ventral margin had one to four teeth, usually three or fewer. On the other hand, *P. yuna* sp. n. had the rostrum slightly curved upward, overreaching the scaphocerite; the ventral margin has two to five teeth, usually three or four. The relative length of the appendix masculina was also useful to distinguish adult individuals of the "Ivonicus/yuna" lineage, since the appendix masculina reached up to 1.5 times the length of the appendix interna in *P. ivonicus* and up to 1.1 times in *P. yuna* sp. n.

Once the validity of *P. ivonicus* and the new species for the Negro River basin were corroborated, we provide illustrations, diagnoses, identification key as well as a description of *P. yuna* sp. n. in order to differentiate the three species from each other as well as from other South American species of *Palaemon*.

Palaemon carteri **(Gordon, 1935)**

Figures 5a and 6a

Palaemonetes carteri Gordon, 1935: 324, fig. 12;–Holthuis 1948: 113;–Holthuis 1950b: 32;–Holthuis 1966: 6 [part, spec. from Rio Tapajós];–Rodríguez 1980: 126;–Rodríguez 1981: 47 [in list];–Rodríguez 1982: 390;–Coelho and Ramos-Porto 1985: 408 [in table];–Holthuis 1993: 8;–Delgado et al. 1997: 16 [in list];–Ramos-Porto and Coelho 1998: 337 [in list];–Barros and Pimentel 2001:

20 [in list];–Vieira 2003: 61;–Magalhães and Pereira 2007: 9, 10, 12 [in list]; Mora-Day et al. 2009: 196 [in list];–Pereira et al. 2010a: 606 [in list];–Pereira et al. 2010b: 84 [in list];–Pileggi et al. 2013: 569 [part, material from Amapá and Bragança; ? material from rio Tapajós basin].

Palaemonetes (*Palaemonetes*) *carteri*–Holthuis 1950c: 10 [in list];–Holthuis 1952: 218, pl. 52, figs c-o, pl. 53, figs a–c;–Holthuis 1959: 81, text-fig. 9;–Kensley and Walker 1982: 11 [part, ? spec. from Rio Curua Una];–López and Pereira 1996: 54, fig. 8;–López and Pereira 1998: 77 [in list]; Melo 2003: 382 [part, not Amazonas].

Holotype. Guyana, upper Cuyuni River, ♂, col. GS Carter (NHM 1935.5.20.19).

Paratypes. Karow Creek, 2 m NE of Penal Settlement, Mazaruni, 1 ♂; River Cuyuni, 1♀ov; Forest Swamp, upper Cuyuni, 1♂; same data as holotype, 7♂ 3♀ 1♀ov 1 juvenile (NHM 1935.5.20.20-29).

Other material. ***Suriname***. *Nickerie*. Lower Naui Kreek, Southern Niew, 10♂ 10♀, col. DC Geijskes, 18 March 1971 (INPA 176). ***Brazil***. *Amapá*. Floresta Nacional do Amapá, igarapé Japim, 5♂ 5♀, col. CRM Santos and JEM Nanzelor, 27 October 2009 (MPEG 1108); Porto Grande, Floresta Nacional do Amapá, tributary of rio Araguari, 1♂ 4♀, col. CRM Santos, 28 October 2009 (CCDB 2755); Macapá, stream in the home of Sr. Marcondes, 1♂ 4♀ov, col. J Cunha, 6 March 2005 (MZUSP 17676). *Pará*. Barcarena, Vila do Conde, 5♂ 2♀ 3♀ov, col. B Mascarenhas, 23 March 2002 (MPEG 739); Belém, Mocambo, 5♂ 5♀ov, col. FR Pimentel and R Maia, January 9 1998 (MPEG 528); Belém, Mocambo, Reserva Mocambo, 4♂ 6♀, col. FR Pimentel and J Dias, 18 June 1999 (MPEG 628); Bragança, Jequeri, Sítio Anacuã, 7♂ 9♀, 23 October 2002 (MPEG 787); Ilha do Marajó, cachoeira do Arari, 1♀, col. J Cunha and J Zuanon, 16 May 2008 (MZUSP 22753); Castanhal, 1♀ov, col. FL Carvalho et al., 14 December 2012 (CCDB 4338); Ilha de Marajó, cachoeira do Arari, rio Arari, igarapé Popudas, 1♂ 1♀, col. J Cunha and J Zuanon, 17 May 2006 (MZUSP 23224); Laranjal do Jari, igarapé Arapiranga, 2♀ 1♀ov, col. Moreira et al., 25 March 2008 (MZUSP 23225); Melgaço, Floresta Nacional de Caxiuanã, 5♂ 4♀, 8 November 1999 (MPEG 717); Santa Maria do Pará, 3 juveniles, col. FL Carvalho et al., 15 December 2012 (CCDB 4339); Tucuruí, rio Tocantins basin, igarapé Santos, 2♀, col. W Zuink and LCF Alvarenga, 16 September 1984 (MNRJ 23382).

Diagnosis. Mandibular palp absent. Rostrum slender, curved upward, reaching or just overreaching the tip of the scaphocerite; dorsal margin with 6 to 10 teeth; ventral margin with 3 to 7 teeth, usually 4 or more. Projection of the anterolateral margin of the first antennular segment overreaching the middle of the second segment, but not reaching, sometimes almost reaching, the dorsal distal margin of the second segment; anterolateral spine of the first antennular segment usually reaches the middle of the projection of the anterolateral margin. Appendix masculina up to 1.1 the length of the appendix interna, measured from their junction. Telson carrying 2 plumose setae between the inner distal stout setae; inner distal stout setae overreaching the distal tip of the telson.

Geographic distribution. Venezuela (Amazonas, Bolívar, Delta Amacuro, Monagas), Guyana, Suriname, French Guiana, Brazil (eastern Amazon: Amapá, Pará).

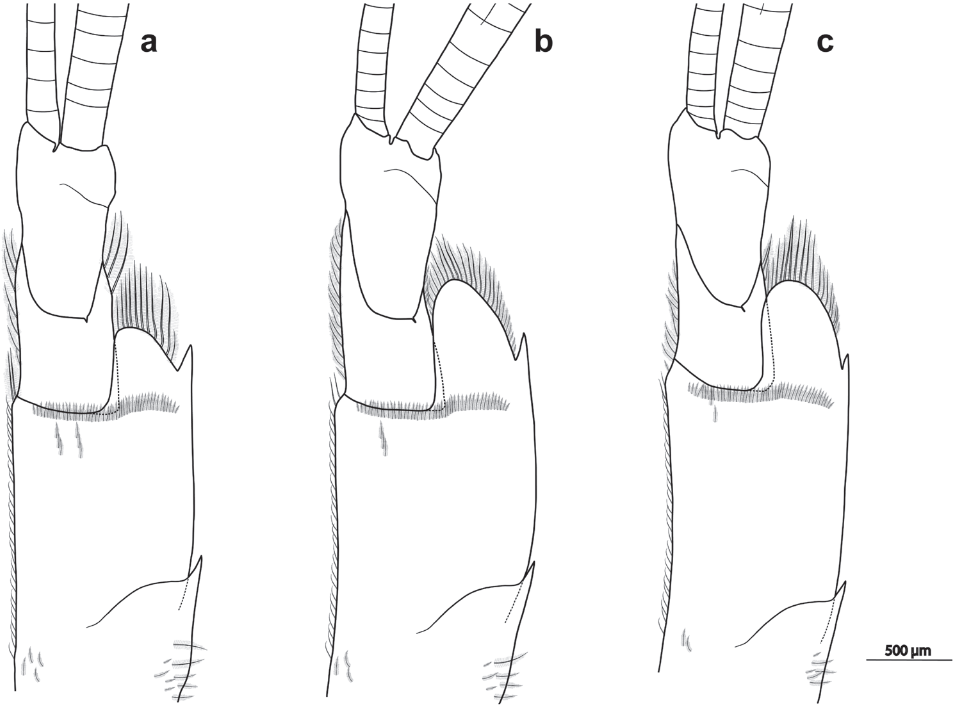

Figure 5. Commonest shape of the antennular peduncle. *Palaemon carteri* (**a** MPEG 787), *P. ivonicus* (**b** INPA 128) and *P. yuna* sp. n. (**c** CCDB 4866).

Ecological features. Usually associated with riparian vegetation, leaf litter and similar microhabitats in lakes, streams and rivers, in areas with low flow. At least in the Amazon river basin, its occurrence is usually associated to clear water river systems.

Palaemon ivonicus (Holthuis, 1950)

Figures 5b and 6b

Palaemonetes ivonicus Holthuis, 1950a: 98;–Holthuis 1966: 4, fig. 1;–Rodríguez 1981: 47 [in list];–Coelho and Ramos-Porto 1985: 408 [in table];–Ramos-Porto and Coelho 1990: 99 [part, not Rio Negro, Catagalo];–Kochalka et al. 1996: 113 [in list];–Ramos-Porto and Coelho 1998: 337 [in list]; Magalhães 1999: 36, 85 [in list];–Magalhães 2000: 59 [in list];–Magalhães 2001: 70, 133 [in list];–Magalhães 2002: 1096, figs 5, 6;–García-Dávila and Magalhães 2003: 675, figs 21–27, 55;–Magalhães and Pereira 2007: 9, 10, 12 [in list];–Valencia and Campos 2010: 224, figs 3–4;–Pileggi et al. 2013: 570.

Palaemonetes carteri–Holthuis 1966: 6 [part, ? 2 spec. from Lago Redondo];–Magalhães 2005: 69, 71 [in list]; Pileggi et al. 2013: 569 [? part, material from rio Xingu and rio Trombetas].

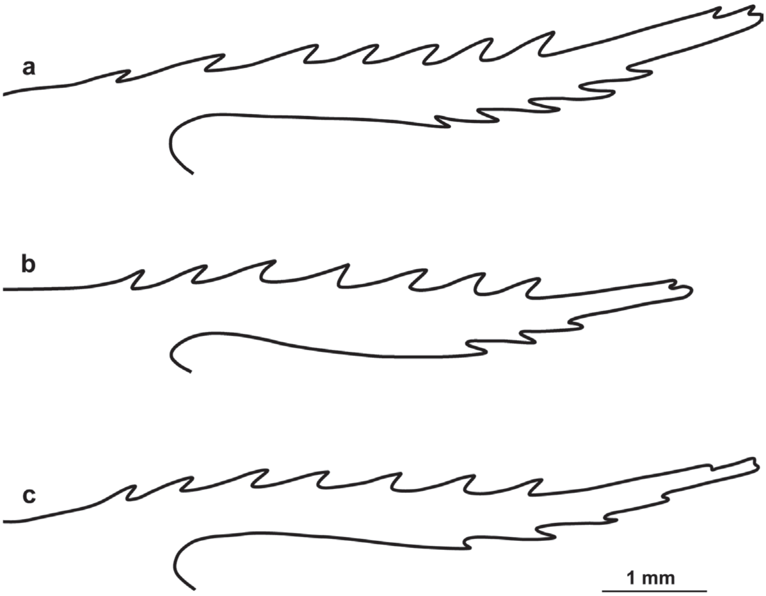

Figure 6. Commonest shape of the rostrum. *Palaemon carteri* (**a** MPEG 787), *P. ivonicus* (**b** INPA 128) and *P. yuna* sp. n. (**c** CCDB 4866).

Palaemonetes (*Palaemonetes*) *ivonicus*–Holthuis 1950c: 10 [in list];–Holthuis 1952: 222, pl. 53, figs d–h;–Melo 2003: 382.

Palaemonetes (*Palaemonetes*) *carteri*–Kensley and Walker 1982: 11 [part., ? spec. from Rio Madeira].

Holotype. Bolivia, Beni, Ivon, Beni River, ♀ (CL 7.5 mm), col. WM Mann, February 1922 (USNM 85234).

Paratype. same data as holotype, 1♀ (CL 6.6 mm) (USNM 85234).

Other material. ***Brazil***. *Acre*. Bujari, igarapé Mapinguari, 2♂ 18♀, col. LR Malabarba et al., 8 August 2001 (UFRGS 3179). *Amazonas*. Itacoatiara, canal Irandiba, 3♂, col. GY Hattori, April 2008 (CCDB 2753); Itacoatiara, igarapé Aeroporto, 2♂ 18♀ (CCDB 4725); Itacoatiara, Poranga, 7♂ 23♀ (CCDB 4632); Itacoatiara, 3♂ 15♀ (CCDB 4716); rio Madeira, Borba, 1♂ 9♀ (MNRJ 1078); rio Solimões, igarapé do Xiboreno, 1♂ 1♀, col. FL Carvalho and EA Souza-Carvalho, 28 January 2012 (CCDB 1435); rio Solimões, lago Janauacá, 1♀, col. J Donnath, 18 March 1978 (MZUSP 8183); rio Solimões, lago do Jacaré, 2♀, col. H Reichardt, 29 March 1967 (MZUSP 6405); Tefé, igarapé da Aeronáutica, 12♂ 8♀, col. JO Chaves, 21 March 1979 (INPA 128). *Pará*. Almeirim, rio Arraiolos, pesqueiro São Paulo, 7♂ 5♀, col. J Carvalho Júnior, 26 July 1999 (MPEG 689); Altamira, rio Xingu, 4♀, col. RM Sousa and Dionísio, 18 December 2000 (MPEG 715); Porto de Moz, rio Xingu, 1♂ 1♀ov, col. R Robles et al., 25 September 2013 (CCDB 4867); rio Tapajós, near the rio Cupari's mouth, downstream Itaiatuba, 1♀, col. C Magalhães and LH Py-Daniel, 27 October 1991 (INPA 1176); Santarém, igarapé do Juá, 2♀, col. LM Sousa and JL Birindelli, 13 November 2006 (MZUSP 28358). *Rondônia*. Rio Guaporé, 1♂ 2♀, col. JC Malta, 25 September 1985 (INPA 326). *Mato Grosso*. Acorizal, 17♂ 6♀, col. Sebastiana, 28 August 1987 (MNRJ 1151); Acorizal, 9♂ 5♀ 4 juveniles, col. Sebastiana (MNRJ 1153); baía do Pio, Pantanal, 5♂ 15 juveniles, col. Sebastiana (MNRJ 1152); Poconé, baía do Pio, 14♂ 33♀ 19 juveniles (INPA 328). *Mato Grosso do Sul*. Rio Negro, córrego Anhumas, 9♂ 11♀, col. C Magalhães et al., 28 August 1998 (CCDB 4667). ***Peru***. *Loreto*. Lago Urcococha, rio Amazonas, 10♂ 5♀, col. C García-Dávila, 10 January 1999 (INPA 883); Quistococha, río Itaya, 12♂ 7♀ov, col. C García-Dávila, 13 July 1998 (INPA 882).

Diagnosis. Mandibular palp absent. Rostrum high, straight or slightly curved upward, not overreaching the scaphocerite; dorsal margin with 6 to 10 teeth; ventral margin with 1 to 4 teeth, usually 3 or fewer. Projection of the anterolateral margin of the first antennular segment reaching or overreaching the dorsal distal margin of the second segment; anterolateral spine of the first antennular segment almost reaching or overreaching the first third of the projection of the anterolateral margin. Appendix masculina up to 1.5 the length of the appendix interna, measured from their junction. Telson carrying 2 plumose setae between the inner distal stout setae; inner distal stout setae overreaching the distal tip of the telson.

Geographic distribution. Venezuela? (Delta Amacuro, Monagas), Colombia? (Amazonas, Arauca, Casanare, Guainía, Meta, Vichada), Brazil (Acre, Amazonas, Mato Grosso, Mato Grosso do Sul, Pará), Bolívia (Beni, Cochabamba, Pando), Peru (Loreto, Madre de Díos), Paraguay.

Ecological features. Usually associated with riparian vegetation, leaf litter and similar microhabitats in lakes, streams and rivers with white or clear water, in areas with low flow. In the western portion of the Amazon river basin it is commonly found in the floodplains of the white water river systems.

Palaemon yuna **sp. n.**

http://zoobank.org/1541C001-E4DD-4812-9D60-277706CE391D

Figures 3, 5c, 6c, 7 and 8

Palaemonetes (*Palaemonetes*) *carteri*–Kensley and Walker 1982: 11, figs 13–14 [part, at least spec. from Rio Negro and its basin]; Melo 2003: 382 [part, Amazonas].

Palaemonetes carteri–Ramos-Porto and Coelho 1990: 99 [Rio Negro, Cantagalo; igarapé afluente do Rio Mapiri, Santarém?];–Pileggi et al. 2013: 569 [? part, material from rio Tapajós basin, rio Xingu and rio Trombetas].

Palaemonetes ivonicus–Ramos-Porto and Coelho 1990: 99 [part, Rio Negro, Cantagalo].

Holotype. Lago Tupé beach, lower Rio Negro tributary, Manaus, Amazonas, Brazil (003°02'42"S, 060°15'10"W), ♂, col. FL Carvalho and EA Souza-Carvalho, 27 January 2012 (CCDB 4865).

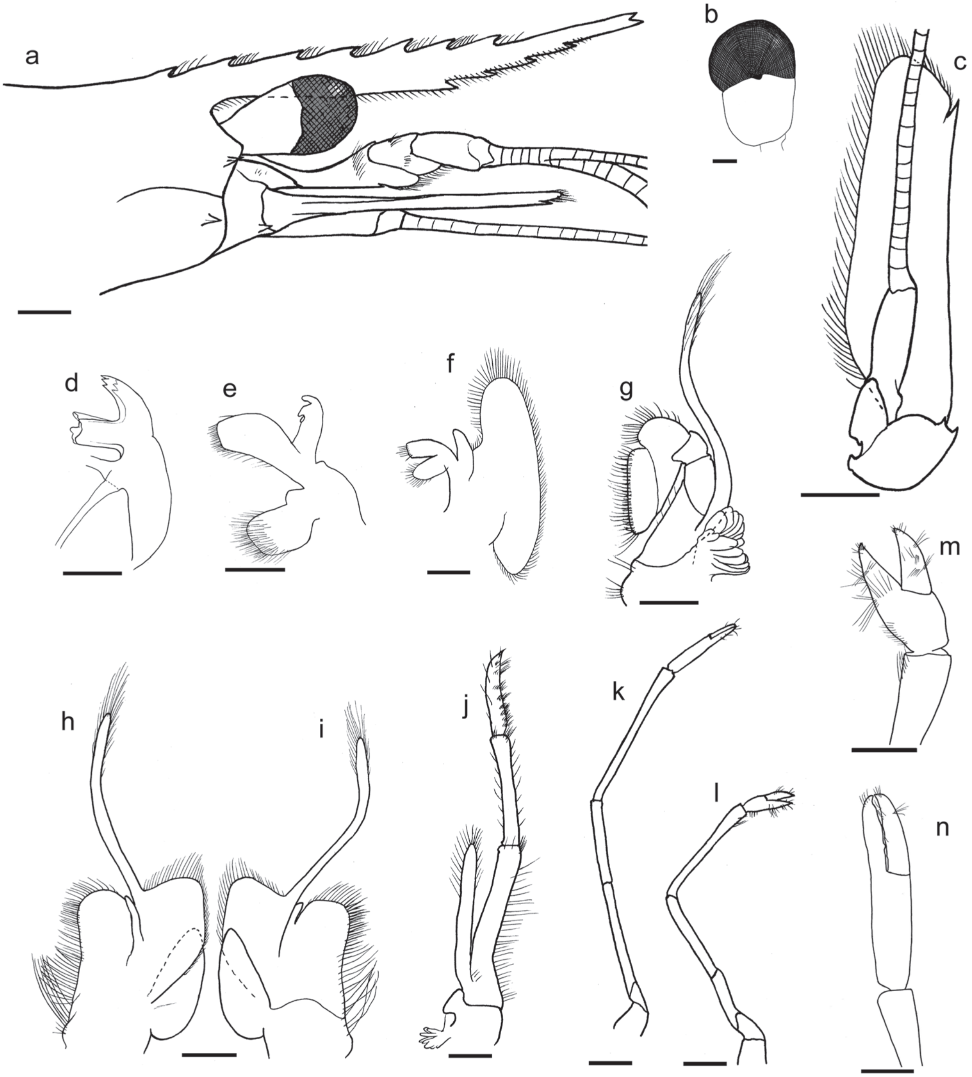

Figure 7. *Palaemon yuna* sp. n. Figure **a** holotype; figures **b–n** paratype (CCDB 4866, male, CL 5.5 mm). **a** anterior part of the carapace **b** right eye, dorsal view **c** left scaphocerite, ventral view **d** left mandible, ventral view **e** left maxillula, ventral view **f** left maxilla, ventral view **g** left second maxilliped, ventral view **h** left first maxilliped, ventral view **i** left first maxilliped, dorsal view **j** right third maxilliped, ventro-lateral view **k** right second pereiopod, ventro-lateral view **l** right first pereiopod, ventro-lateral view **m** right first chela, mesial view **n** right second chela, mesial view. Scale bar: **a, c, k** equal to 1 mm; others equal to 0.5 mm.

Paratypes. same data as holotype, 28♂ 8♀ 17♀ov (CCDB 4866); same data as holotype, 10♂ 3♀ 3♀ov (INPA 2016); same data as holotype, 1♂ 1♀ 1♀ov (OUMNH-ZC 2013-08-001).

Other material. ***Brazil***. *Amazonas*. Parque Nacional de Anavilhanas, lake near the rio Apuaí's mouth, 1♂ 1♀, col. J Zuanon, 20 August 2005 (INPA 1432); Manaus, Rio Negro basin, Igarapé do Camarão, 20♀ov, col. O Odinetz-Collart et al., 28 February 1989 (CCDB 4726); Rio Negro basin, igarapé Alagadiço, 20♂, col. O Odinetz-Collart et al., 17 January 1989 (CCDB 4727); São Gabriel da Cachoeira, igarapé Barixia, right bank of the Rio Negro, 4♀, col. J Cunha et al., 14 December 2005 (MZUSP 16907); Santa Izabel do Rio Negro, 2♀, 24 October 1972 (MZUSP 13645); rio Uatumã, near the igarapé do Miriti's mouth, 1♂ 3♀, col. C Magalhães, 12 July 1985 (INPA 173).

Diagnosis. Mandibular palp absent. Rostrum slender, slightly curved upward at the distal half, overreaching the scaphocerite; dorsal margin with 6 to 10 teeth; ventral margin with 2 to 5 teeth, usually 3 or 4. Projection of the anterolateral margin of the first antennular segment reaching the dorsal distal margin of the second segment; anterolateral spine of the first antennular segment not overreaching the first third of the projection of the anterolateral margin. Appendix masculina up to 1.1 the length of the appendix interna, measured from their junction. Telson carrying 2 plumose setae between the inner distal stout setae; inner distal stout setae overreaching the distal tip of the telson.

Description. Carapace glabrous. Sub-orbital lobe and pterygostomial angle rounded. Branchiostegal suture located approximately with a half of the distance between the antennal and branchiostegal tooth. Branchiostegal tooth almost as strong as the antennal, placed behind the anterior margin of the carapace.

Rostrum slender, slightly curved upward at the distal half, overreaching the scaphocerite (Fig. 7a); dorsal margin with 6 to 10 teeth, 5 to 8 of them placed in the proximal 2/3, 1 or 2 dorsal teeth located behind the orbit, 1 or 2 subapical teeth; ventral margin with 2 to 5 teeth, usually 3 or 4, all of them placed in the distal half. Single row of setae present on the proximal ventral portion up to the second tooth; double continuous and uniformly spaced row of setae on the distal half.

Eye well developed with pigmented cornea (Fig. 7b); cornea slightly wider and smaller than the eyestalk; ocellus present on dorsal side.

Antennular peduncle not reaching the distal margin of the scaphocerite; first antenular segment with outer margin slightly convex and projection of the anterolateral margin rounded, reaching the dorsal distal margin of the second segment (Fig 5c); anterolateral spine of the first antennular segment not overreaching the first third of the projection of the anterolateral margin; second segment as broad as and shorter than the third segment; inner ventromesial tooth present; upper antennular flagellum fused for about 1/5 of its length (4–6 segments fused, 14–16 free); free portion with two rows of two or three aesthetascs on each segment. Stylocerite short, not reaching the middle of the first antennular segment. Béc ocellaire with anterior margin concave, pronounced upwardly and bearing a pigmented spot dorsally.

Scaphocerite slender (Fig. 7c), laminar, 3.3 times as long as broad; outer margin slightly concave, terminating in a tooth, not overreaching the lamella; basal segment of antenna with strong lateral tooth. Flagellum of the antenna more than five times the length of the body.

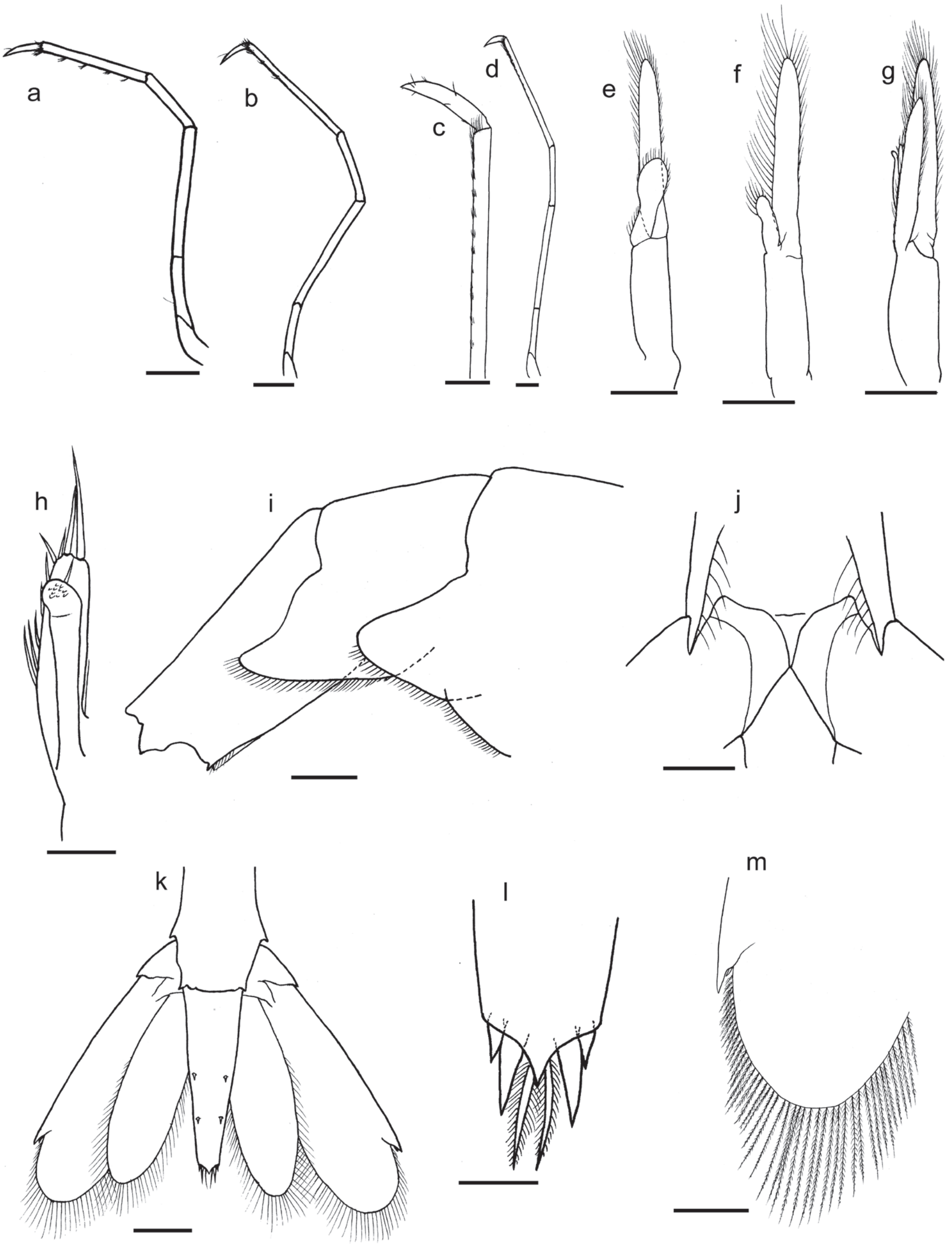

Figure 8. *Palaemon yuna* sp. n. Figures **i** and **m** holotype; figures **a–e, g, h, j–l** paratype (CCDB 4866, male, CL 5.5 mm); figure **f** paratype (CCDB 4866, female, CL 5.5 mm). **a** left third pereiopod, lateral view **b** left fourth pereiopod, lateral view **c** distal portion of the left fifth pereiopod, lateral view **d** left fifth pereiopod, lateral view **e** left first pleopod, posterior view **f** left first pleopod, posterior view **g** left second pleopod, posterior view **h** left appendix masculina and appendix interna, posterior view **i** right posterior part of the abdomen, lateral view **j** pre-anal plate, ventral view **k** telson and uropods, dorsal view **l** distal part of the telson, dorsal view **m** left distal portion of the exopod of the uropod, dorsal view. Scale bar: **a, b, d–g, i, k** equal to 1 mm; **c, j, m** equal to 0.5 mm; **h, l** equal to 0.25 mm.

Mandibular palp absent; incisor process with three teeth on both sides (Fig. 7d). Upper lacina of the maxillula just reaching the length of the inner lacina (Fig. 7e). First maxilliped with lobes of the epipod fused (Fig. 7h–i); anterior lobe elongated and almost triangular, with lateral border slightly convex; junction between the endites roundly curved. Epipod of the third maxilliped with anterior margin forming an angle of about 45° (Fig. 7j).

Thoracic sternal armature sexually similar. First thoracic sternite with an acute tooth and a conspicuous transverse ridge; second without tooth, bearing a triangular transverse ridge. Third to fifth without tooth and ridge incomplete.

First pereiopod slender (Fig. 7l), reaching the tip of scaphocerite with the fingers; ischium 1.8 times the length of basis; merus 1.7 times the length of ischium; carpus 1.3 times the length of merus; chela slightly less than 0.5 the length of carpus; fingers as long as palm (Fig. 7m).

Second pereiopod slender (Fig. 7k), overreaching the scaphocerite with about a half of the inflated distal part of the carpus; ischium about 4.5 times the length of basis; merus 0.8 the length of ischium; carpus 1.8 times the length of merus; chela about 0.6 times the length of carpus; fingers about 0.7 the length of palm (Fig. 7n).

Third pereiopod (Fig. 8a) slender, reaching the tip of the scaphocerite; ischium 1.8 times the length of basis; merus 1.9 times the length of ischium; carpus about 0.5 times the length of merus; propodus 1.6 times the length of carpus, ventral margin armed with 5–8 cuspidate setae; dactylus simple, about 0.3 times the length of the propodus.

Fourth pereiopod slender (Fig. 8b), overreaching the scaphocerite with all length of the dactylus; ischium 1.7 times the length of basis; merus 2.4 times the length of ischium; carpus about 0.5 length of merus; propodus 1.7 times the length of carpus, ventral margin armed with 7-13 cuspidate setae; dactylus simple, about 0.3 the length of propodus.

Fifth pereiopod slender (Fig 8c-d), overreaching the scaphocerite with the end of the propodus; ischium 1.7 times the length of basis; merus 2.3 times the length of ischium; carpus about 0.6 length of merus; propodus 2.1 times the length of carpus; grooming brush comprises about 10 rows of setae on the distal third of the propodus; dactylus simple, about 0.2 the length of propodus.

First pleopod without appendix interna and sexually dimorphic in proportions; males with endopod 0.5 the length of exopod (Fig. 8e); females with endopod approximately 0.3 length of exopod (Fig. 8f). Second to fifth pleopods similar, with the endopod reaching about 0.8 the length of exopod and bearing an appendix interna (Fig. 8g). Appendix masculina up to 1.1 times the length of the appendix interna, measured from their junction (Fig. 8h).

Abdominal sternal armature sexually dimorphic; males with first and second sternites bearing median process; second more acute and bigger than the first process; females with median process less develop than males.

Abdominal pleura furnished with plumose setae on ventral margin; fifth pleuron elongated and disto-ventrally rounded (Fig. 8i), with dorsal posterior border concave; sixth segment 1.63 times the length of the fifth; posterolateral margin with small tooth and keel disto-ventrally. Anal plate unarmed (Fig. 8j).

Figure 9. Type locality of *Palaemon yuna* sp. n. Lago Tupé beach, lower Rio Negro tributary, Manaus, Amazonas, Brazil (003°02'42"S, 060°15'10"W).

Telson as long as sixth pleonite; dorsal surface with two pairs of cuspidate setae (Fig. 8k); proximal dorsal tuft of setae reduced to one or two simple setae; marginal setae absent; posterior margin ending abruptly in a triangular tip, not overreaching the inner stout setae (Fig. 8l); 1 pair of plumose setae and 2 pairs of stout setae, inner pair of stout setae about 2.3 times the length of the outer pair.

Uropods overreaching the telson by 0.3 of the length of exopod; exopod 1.25 times the length of endopod; mobile distolateral setae of exopod weak, reaching about the middle of the fixed tooth (Fig. 8m).

Geographic distribution. Brazil (Amazonas, Pará?), Venezuela? (Apure).

Ecological features. Usually associated with riparian vegetation, leaf litter and similar microhabitats in lakes, streams and rivers of black or clear water river systems, in areas with low flow.

Etymology. The specific epithet is derived from the Tupi, the general language of the Brazilian indigenous people: *y* = water, river + *úna* = black, alluding to the environment where the species was first found (Fig. 9).

Key for the species of the *Palaemon carteri* / *P. ivonicus* / *P. yuna* sp. n. group

1 Projection of the anterolateral margin of the first antennular segment not overreaching the middle of the second segment .... **other South American Palaemoninae**

– Projection of the anterolateral margin of the first antennular segment overreaching the middle of the second segment **2**

2 Mandibular palp present.................. **other South American Palaemoninae**

– Mandibular palp absent.. **3**

3 Projection of the anterolateral margin of the first antennular segment not reaching, rarely almost reaching, the dorsal distal margin of the second segment; anterolateral spine of the first antennular segment usually reaching the middle of the projection of the anterolateral margin (Fig. 5a); rostrum with 3 to 7 ventral teeth (usually 4 or more)............................... ***Palaemon carteri***

– Projection of the anterolateral margin of the first antennular segment reaching or overreaching the dorsal distal margin of the second segment; anterolateral spine of the first antennular segment not reaching the middle of the projection of the anterolateral margin (Fig. 5b–c); rostrum with 1 to 5 ventral teeth (usually 4 or fewer).. **4**

4 Rostrum high, straight or slightly curved upward, not overreaching the scaphocerite; rostrum with 1 to 4 ventral teeth (usually 3 or fewer) (Fig. 6b). Anterolateral spine of the first antennular segment generally reaching the first third of more of the projection of the anterolateral margin (Fig. 5b)............ .... ***Palaemon ivonicus***

– Rostrum slender, slightly curved upward, overreaching the scaphocerite; rostrum with 2 to 5 ventral teeth (usually 3 or 4) (Fig. 6c). Anterolateral spine of the first antennular segment generally not reaching the first third of the projection of the anterolateral margin (Fig. 5c).......... ***Palaemon yuna* sp. n.**

Discussion

Both molecular and morphological data support the validity of *P. ivonicus*, refuting the hypothesis that this species is a junior synonym of *P. carteri*. The two species are allocated in two sisters Amazonian lineages, with great genetic divergence and morphological support. Additionally, a third species closely related to *P. ivonicus* is described.

The projection of the anterolateral margin and anterolateral spine of the first antennular segment seems to be an important character in this group, although we have found some specimens with a state of character close to an intermediate form, making difficult a clear distinction between these lineages. Additionally, there is an ontogenetic variation, which needs to be considered in the analyses. Some large specimens of the “Carteri” lineage have the antennular projection almost reaching the dorsal margin of the second segment. On the other hand, some small specimens of the “Ivonicus/yuna” lineage also have the projection almost reaching the dorsal margin of the second segment. However, even considering those limitations, the antennular character was the most constant one to distinguish the “Carteri” and “Ivonicus/yuna” lineages. Therefore, we suggest that this character must be evaluated in further morphological analyses within the *Palaemon* genus.

The wide intraspecific morphological variability and interspecific similarity between *P. carteri* and *P. ivonicus* as well as the presumptive synonymy have been reported by previous studies since the 1970s (Gomes-Corrêa 1977, Odinetz-Collart and Enriconi 1993, García-Dávila and Magalhães 2003, García-Dávila et al. 2005). Despite of that similarity posteriorly reported, Holthuis (1950a, 1952) did not make a direct comparison of *P. carteri* and *P. ivonicus* in order to clearly differentiate them. Furthermore, the type series of *P. ivonicus* is composed by only two specimens, making it impossible to evaluate the morphological variability spectrum within this species. Therefore, it is not surprising that misidentification may occur, which may have contributed to the hypothesis of synonymy.

The overlapping of several morphological characters demonstrated by Odinetz-Collart and Enriconi (1993) and García-Dávila et al. (2005) probably is due to the fact that all or most of the specimens examined by these authors were from the "Ivonicus/yuna" lineage. These studies used the traditional rostral characters to identify some specimens from the central Amazon basin as *Palaemon carteri*. We were unable to find any sample of *P. carteri* in the central and western Amazon basin, since specimens from these regions are more likely to be assigned to the "Ivonicus/yuna" lineage. Therefore, the rostral similarity between *P. carteri* and *P. yuna* sp. n. probably has led many authors to use the name *P. carteri,* which was the name available at the moment that better fitted the rostral characteristics of some populations from the central Amazon basin (see synonymic list of *P. yuna* sp. n.).

The wide genetic dissimilarity between the "Carteri" and "Ivonicus/yuna" lineages shows that they have no recent divergence, as one could expect based only on their morphological similarity. The approximately 10 million years of divergence estimated for these lineages, based on the 16S rRNA gene, may be associated with marine incursion as well as colonization of different environments in western Amazonia during the Middle Miocene (~16 to 11.6 Ma) and Late Miocene (~11.6 to 5.3 Ma) (Fig. 10). From the Early Miocene until the early Late Miocene (~23 to 9 Ma), the western Amazonia region was mostly submerged and transformed into a continually shifting mosaic of lakes, wetlands and river belts (Wesselingh 2006), which are similar to the environments where specimens of the "Ivonicus/yuna" lineage are currently found. This continually shifting mosaic, the Pebas system, had contact with the Caribbean Sea and underwent several marine incursions during the Middle and Late Miocene (Hoorn 1993, Wesselingh 2006). Particularly in the period between 11.8 and 10 Ma, close to our estimate for the time of divergence between the "Carteri" and "Ivonicus/yuna" lineages, there is evidence of an extensive marine transgression into the low-lying basins of South America (Lundberg et al. 1998). These marine incursions, which established brackish-water conditions in the late Middle Miocene and early Late Miocene (Hoorn 1993, Lundberg et al. 1998), might have isolated freshwater lineages of the Pebas system from others distributed in northern South America outside this wetland system, where the "Carteri" lineage is currently found (Fig. 11). Similar biogeographical patterns and speciation events associated with the Pebas system and marine incursions in the Middle and Late Miocene have been reported for several groups in the Amazon region (Hoorn 1993, Lovejoy et al. 1998, 2006, Lundberg et al. 1998, Wesselingh 2006, Santos et al. 2009, Cooke et al. 2012a, b). Therefore, the proposed

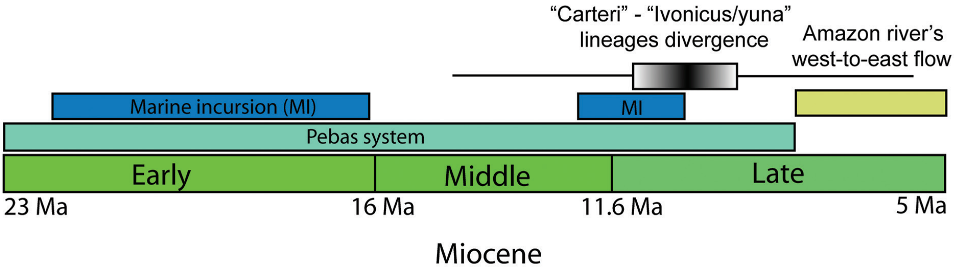

Figure 10. Historical context for the proposed divergence time between the "Carteri" and "Ivonicus/yuna" lineages.

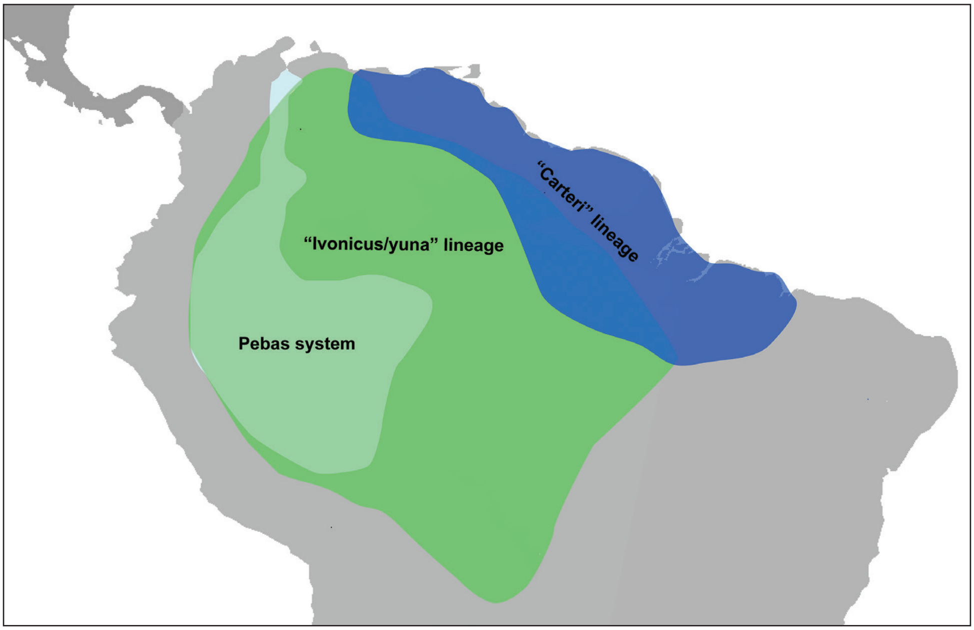

Figure 11. Putative current distribution of the "Carteri" and "Ivonicus/yuna" lineages. [Pebas system during the Late Miocene (~11.8 to 10 Ma) according to Lundberg et al. 1998.]

current parapatric distribution of the "Carteri" and "Ivonicus/yuna" lineages probably developed after the establishment of the modern west-to-east course of the Amazon River, which may have initiated approximately 8 Ma (Lundberg et al. 1998).

The specimens from the Negro River (*P. yuna* sp. n.) have considerable genetic divergence from the specimens collected in the Solimões-Amazon and Xingu rivers, being allocated outside the *P. ivonicus sensu stricto* clade. The specimens from the Negro River also show some differences in the rostrum, in the antennular characters and in the appendix masculina compared to specimens from the type locality of *P. ivonicus* as well as other basins (Figs 5–6). Additionally, a morphometric study with specimens from the Negro River revealed that they form a morphometrically distinct group from populations that inhabit white water, although some overlapping was found (García-Dávila et al. 2005). Studies with fishes reported a similar pattern, providing evidence for the effect of divergent natural selection associated with the difference in water colour between the Negro River and Solimões-Amazon River (Cooke et al. 2012a,b). Genetic divergence between hydrologically different, but interconnected, environments has been reported for other palaemonid species without a conspicuous and consistent morphological differentiation (Carvalho et al. 2013). However, the characters described above allow the morphological distinction between *P. ivonicus* and *P. yuna* sp. n. Therefore, regarding the genetic, morphological and ecological differentiation, there is sufficient evidence to justify the proposal of a new species for the populations from the Negro River basin. The actual distributional range of *P. yuna* sp. n. may be wider than indicated by the material available for this study. Specimens from the lower Tapajós River basin might be co-specific with the specimens from the Negro River basin, as suggested by the results of a molecular study conducted by García-Dávila (2002) in an unpublished academic thesis, using the mitochondrial cytochrome c oxidase subunit I gene (COI).

Specimens from the upper Orinoco River basin have antennular characters similar to those of *P. yuna* sp. n. Nevertheless, some specimens fail to have a curved upward rostrum overreaching the scaphocerite. The connection between the Negro and Orinoco river basins through the Casiquiare River opens the possibility of the existence of a conspecific group occurring in the Negro and Orinoco rivers. A similar biogeographical pattern has been reported for fish species, using molecular data (Willis et al. 2010). However, despite logistic difficulties, a more thorough genetic and morphological sampling along the Negro River and the upper Orinoco River basins should be carried out in order to verify the phylogenetic relationships among the populations from the upper Orinoco and other populations of the “Ivonicus/yuna” lineage as well as its taxonomic status. Similarly, the records of *P. ivonicus* from the Orinoco River basin (López and Pereira 1996, 1998, Pereira et al. 2010a, b) should be verified, since morphologically (judged by the illustration provided by López and Pereira 1996: 55, fig. 9) and zoogeographically these specimens probably do not belong to this taxon.

The occurrence of *P. ivonicus* in the Paraguay/lower Paraná River basin is an issue that needs further analyses in order to verify the phylogenetic relationships of these populations. As discussed by Magalhães et al. (2005), the decapod fauna of the Amazon and the Paraguay-Paraná river lowlands has several common elements whose current distributions may be result of dispersal across the paleobasins of these systems during Tertiary and Quaternary as some geological events changed their boundaries promoting different sequences of capture of headwater (Lundberg et al. 1998). Even in recent times, sporadic or seasonal contact between the Amazon and the Paraguay-Paraná fluvial nets can occur, as some landscape features along their boundary favor transfluences, headwater captures, floods and spillouts to one or other side (Iriondo and Paira 2007).

An additional record of *P. ivonicus* from the São Francisco River basin, state of Minas Gerais, Brazil (Melo 2003), cannot be confirmed as it was not documented by voucher material, and we were unable to find any samples from this basin in the collections visited by us. In addition, we could not find any individual of this species in field collections made in that state. Moreover, the geological history of the São Francisco River basin does not show any evidence of a connection with the Amazonian basin during the Neogene or Quaternary period, which makes unlikely a natural occurrence of *P. ivonicus* in this basin. The report of *P. carteri* for Mexico (Felder et al. 2009) seems to be an error, since the notes associated to the species do not match with the distribution and habitat of the species, and no other studies have reported this species for the Gulf of Mexico.

The fact that our target group seems to be closer related to three neotropical species of *Palaemon* (*P. gracilis*, *P. hancocki* and *P. pandaliformis*) than to other species of the genus require further studies. Ashelby et al. (2012) recovered *P. gracilis* and *P. pandaliformis* outside the clade that comprised the majority of the species of *Palaemon*. Therefore, *P. carteri*, *P. ivonicus* and *P. yuna* might also not have a close relationship with *Palaemon sensu stricto*. However, as this was not the goal of the present study, a broader taxonomic sampling of the subfamily using both mitochondrial and nuclear genes is needed to address properly these generic-level questions.

Our study is part of a project aiming to investigate the American species of *Palaemon*, and this is the first one which uses a multidisciplinary approach aiming to clarify this taxonomic issue. Our data clearly show that there are at least two morphologically and genetically distinct lineages, which might have diverged ~10 Ma. A multilocus approach is needed to provide more molecular support for this estimated divergence time. The possibility of hybridization cannot be rejected and must be deeply investigated in further studies. Moreover, the morphological variability found in some populations still assigned to *P. ivonicus* as well as the molecular variability found within the "Carteri" lineage need to be further investigated to verify whether there are other morphologically similar species not yet described.

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RESEARCH ARTICLE

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Molecular perspective on the American transisthmian species of *Macrobrachium* (Caridea, Palaemonidae)

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Abstract

The closure of the Isthmus of Panama (about 3.1 million years ago) separated previously continuous populations and created two groups of extant species, which live now in the Pacific and Atlantic drainage systems. This relatively recent event was a trigger to diversification of various species in the Neotropics, nonetheless there are exemplars that do not show sufficient morphologic variability to separate them by traditional morphological tools. About 60 years ago, some freshwater decapod species with high morphological similarity were separate by previous researchers, based on geographical distribution, in Pacific and Atlantic and considered as "sister species". However, the complete isolation of these prawns by this geographical barrier is questionable, and it has generated doubts about the status of the following transisthmian pairs of sibling species: *Macrobrachium occidentale* × *M. heterochirus*, *M. americanum* × *M. carcinus*, *M. digueti* × *M. olfersii*, *M. hancocki* × *M. crenulatum*, *M. tenellum* × *M. acanthurus* and *M. panamense* × *M. amazonicum*. Here we evaluated the relation among these pairs of sibling species in a molecular phylogenetic context. We generated 95 new sequences: 26 sequences of 16S rDNA, 25 of COI mtDNA and 44 of 18S nDNA. In total, 181 sequences were analyzed by maximum likelihood phylogenetic method, including 12 *Macrobrachium* transisthmian species, as well as seven other American *Macrobrachium* species, and two other palaemonids. Our analysis corroborated the morphological proximity of the sibling species. Despite the high degree of morphological similarities and considerable genetic diversification encountered among the transisthmian sister species, our data support the conclusion that all species included in sibling groups studied herein are valid taxonomic entities, but not all pairs of siblings form natural groups.

Keywords

Freshwater decapods, genetic variability, molecular phylogeny, Palaemoninae, sibling species

Introduction

In the late Pliocene, the closure of the Isthmus of Panama was a trigger to the diversification of many species in the Neotropics. The separation of previously continuous populations created two groups of extant species, which live now in the Atlantic and Pacific drainage systems. This vicariant event opened a unique opportunity for studies on evolution, divergence and speciation processes (Knowlton et al. 1993, Knowlton and Weigt 1998, Lessios 2008). The Central American land bridge is a well-dated biogeographic barrier and is a relatively recent event, about 3.1 million years ago (Keigwin 1978, Coates et al. 1992, Coates and Obando 1996, Anger 2013). Since then, the Atlantic and Pacific marine ecosystems became gradually separated, whereas the gene flow was blocked between organisms on either side.

In spite of the geographic separation, some species are difficult or impossible to distinguish using traditional morphological features, and are thus called "sibling species" (see Knowlton 1993 and references cited therein). These sibling species refer to pairs of species that are genetically closely related, but reproductively isolated (Mayr 1963, Steyskal 1972, Knowlton 1986). Others authors refer to "sibling" as "geminate species" (Jordan 1908, Stillman and Reeb 2001, Marko 2002), in which individuals were separated necessarily by a geographic barrier, and each member of the pair occurs along one coast of the Americas (Lessios 1998, Miura et al. 2010). Other non-morphological features have been used to distinguish these species such as "karyology, hybridization experiments to detect postzygotic incompatibility, distribution patterns, resource use, breeding season, life history and development, mating behavior (including visual, acoustical, and chemical signals), color pattern, and various biochemical characters" (Knowlton 1986). Consequently, a pair of species, reproductively isolated and very similar in morphology, is not necessarily considered as sibling species, and an interdisciplinary approach is necessary to evaluate this conclusion.

Molecular tools have been used to contribute with species delimitation in several cryptic decapods (Schubart et al. 2001a, b, Kitaura et al. 2002, Lai et al. 2010, Pileggi and Mantelatto 2010, Mantelatto et al. 2011, Negri et al. 2012, Torati and Mantelatto 2012). Phylogenies based on molecular data has evidenced probable cases of misidentification of sibling species based on morphology (Lessios 2008, Rossi and Mantelatto 2013). For some freshwater species, the isolation by the closure of the Isthmus of Panama might be questionable, since species of the genus *Macrobrachium* Spence Bate, 1868 can disperse over greater distances than the width of the Isthmus (Steeves et al. 2005, Bauer and Delahoussaye 2008, Bauer 2011) and may also use the Panama Canal as passageway for both sides (Hildebrand 1939, Abele and Kim 1989).

Most studies on decapods sister species focused only in marine species of the genus *Alpheus* Fabricius, 1798 (Knowlton et al. 1993, Knowlton and Weigt 1998, Wehrtmann and Albornoz 2002), while our knowledge of the impact of the Isthmus of Panama on freshwater-invading decapods is extremely limited (Anger 2013). Prawns of the genus *Macrobrachium* are widely distributed in rivers of tropical and subtropical regions with more than 240 recognized species worldwide (De Grave and Fransen 2011). Although its greatest diversity has been found in the Indo-Pacific region, in the Americas there are more than 55 valid species, representing an area of great importance concerning the diversity of the family Palaemonidae (Holthuis 1952, Pileggi and Mantelatto 2012).

The high morphological similarity between some American species led Holthuis (1952) to designate Atlantic and Pacific *Macrobrachium* "sister species". Until now, morphological similarities between the transisthmian "sibling species" have impeded the identification of the following pairs of species: *Macrobrachium occidentale* × *M. heterochirus*, *M. americanum* × *M. carcinus*, *M. digueti* × *M. olfersii*, *M. hancocki* × *M. crenulatum*, *M. tenellum* × *M. acanthurus* and *M. panamense* × *M. amazonicum*. These species occur primarily in Central America, with the first species of each pair is found in the Pacific drainage and the second in the Atlantic side. Larvae of these species require saline water (*i.e.*, 10–35 ppt) to complete their life cycle, and exhibit other adaptive features, such as extended larval development and amphidromous life histories (Hedgpeth 1949, Bauer and Delahoussaye 2008, Bauer 2011, 2013). Moreover, these prawns show great morphological modifications during ontogenesis, and as other congeneric species they present controversial systematic issues, with high interspecific conservatism and males with intraspecific variation, as found among distinct morphotypes (Holthuis 1952, Moraes-Riodades and Valenti 2004, Pileggi and Mantelatto 2010, Vergamini et al. 2011). Considering the doubt whether the previously indicated species of Central American *Macrobrachium* are sister taxa or not, our study aimed to evaluate in a molecular phylogenetic context the relationships among 12 transisthmian *Macrobrachium* "sibling species" from the Americas in order to assess the validity of their current species level.

Methods

Sample collection

Fresh specimens for molecular analysis were obtained from field collections in rivers and estuaries in Brazil, Chile, Venezuela, and Costa Rica (Table 1). The individuals were preserved in 75–90% ethanol and deposited in the Crustacean Collection of the Department of Biology (CCDB), Faculty of Philosophy, Sciences and Letters at

Table 1. Trans-isthmian species of *Macrobrachium* and other palaemonids used for the phylogenetic analyses, with the respective collection locality, distribution of the species, catalogue number, and genetic database accession numbers at GenBank.

Species	Locality	Distribution	Catalogue Nº	16S	COI	18S
Sibling species of *Macrobrachium*						
M. acanthurus-1	Ilha de São Sebastião-SP, Brazil	America-Atlantic	CCDB 2134	HM352445	HM352485	KM101492
M. acanthurus-2	Guaraqueçaba-PR, Brazil	America-Atlantic	CCDB 2546	HM352444	KM101538	KM101493
M. acanthurus-1	Puerto Viejo, Costa Rica	America-Atlantic	CCDB 1556	KM101464	KM101537	KM101491
M. acanthurus-2	Cahuita, Costa Rica	America-Atlantic	CCDB 2901	KM101465	KM101539	KM101494
M. acanthurus-1	Bocas del Toro, Panama	America-Atlantic	CCDB 3538	KM101467	KM101541	KM101496
M. acanthurus-2	Panama	America-Atlantic	CCDB 3536	KM101466	KM101540	KM101495
M. tenellum-1	Puntarenas, Costa Rica	North/Central America-Pacific	MZUCR 1936-002	KM101488	KM101567	KM101534
M. tenellum-2	Guanacaste, Costa Rica	North/Central America-Pacific	MZUCR 3290-01	KM101489	KM101568	KM101535
M. tenellum	Oaxaca, Mexico	North/Central America-Pacific	CNCR 24831	KM101487	KM101566	KM101533
M. amazonicum-1	Santana-AP, Brazil	South/Central America-Atlantic	CCDB 1965	HM352441	HM352486	KM101497
M. amazonicum-2	Aquidauana-MS, Brazil	South/Central America-Atlantic	CCDB 1970	HM352442	HM352487	-
M. amazonicum-3	Itacoatiara-AM, Brazil	South/Central America-Atlantic	CCDB 2085	HM352443	HM352488	-
M. amazonicum	Panama	South/Central America-Atlantic	CNCR 5151	KM101468	KM101542	KM101498
M. panamense-1	Cerca Camaronera, Costa Rica	Central America-Pacific	MZUCR 2972-01	KM101485	KM101562	KM101528
M. panamense-2	Río Tempisque, Costa Rica	Central America-Pacific	MZUCR 2971-01	KM101484	KM101561	KM101527
M. panamense-3	Guanacaste, Costa Rica	Central America-Pacific	MZUCR 3291-01	KM101486	KM101563	KM101529
M. olfersii-1	Ilha de São Sebastião-SP, Brazil	America-Atlantic	CCDB 2435	HM352459	HM352496	KM101523
M. olfersii-2	Antonina-PR, Brazil	America-Atlantic	CCDB 2445	HM352458	KM101558	KM101524
M. olfersii	Isla Margarita, Venezuela	America-Atlantic	CCDB 2446	HM352460	KM101559	KM101525
M. olfersii-1	Reserva Veragua, Costa Rica	America-Atlantic	CCDB 4873	KM101483	KM101560	KM101526
M. olfersii-2	Costa Rica (Atlantic)	America-Atlantic	CCDB 2876	JQ805835	JQ805933	JQ805858
M. olfersii-3	Costa Rica (Atlantic)	America-Atlantic	CCDB 2880	JQ805839	JQ805936	JQ805859
M. digueti-1	Costa Rica (Pacific)	South/Central America-Pacific	CCDB 2882	JQ805806	JQ805903	JQ805847
M. digueti-2	Costa Rica (Pacific)	South/Central America-Pacific	CCDB 3091	JQ805807	JQ805904	-
M. digueti-3	Río Aranjuez, Costa Rica	Central America-Pacific	MZUCR 3292-01	KM101476	KM101551	KM101514

Species	Locality	Distribution	Catalogue Nº	16S	COI	18S
M. digueti	Mexico	South/Central America-Pacific	CNCR 24811	JQ805808	JQ805906	JQ805849
M. crenulatum-1	Isla Margarita, Venezuela	South/Central America-Atlantic	CCDB 2124	HM352463	HM352498	KM101512
M. crenulatum-2	Venezuela	South/Central America-Atlantic	IVIC 123	JQ805801	-	JQ805845
M. crenulatum-1	Costa Rica	South/Central America-Atlantic	CCDB 2873	JQ805804	JQ805900	JQ805846
M. crenulatum-2	Costa Rica	South/Central America-Atlantic	CCDB 2877	JQ805800	-	JQ805844
M. crenulatum-3	Reserva Veragua, Costa Rica	South/Central America-Atlantic	CCDB 4874	KM101475	KM101550	KM101513
M. hancocki-1	Costa Rica	South/Central America-Pacific	CCDB 3090	JQ805813	JQ805911	-
M. hancocki-2	Costa Rica	South/Central America-Pacific	CCDB 3092	JQ805814	JQ805912	JQ805851
M. hancocki-3	Costa Rica	South/Central America-Pacific	CCDB 3757	JQ805821	JQ805920	-
M. hancocki-4	Costa Rica	South/Central America-Pacific	CCDB 3756	JQ805822	JQ805919	-
M. hancocki	Panama	South/Central America-Pacific	RMNHD 8810	JQ805817	JQ805915	JQ805852
M. carcinus-1	Santana-AP, Brazil	America-Atlantic	CCDB 2122	HM352448	HM352490	KM101507
M. carcinus-2	Ubatuba-SP, Brazil	America-Atlantic	CCDB 2136	HM352449	HM352491	KM101509
M. carcinus	Isla Margarita, Venezuela	America-Atlantic	CCDB 2123	HM352450	HM352492	KM101508
M. carcinus-1	Río Suarez, Costa Rica	America-Atlantic	CCDB 2145	HM352452	KM101548	KM101510
M. carcinus-2	Cahuita, Costa Rica	America-Atlantic	CCDB 4876	KM101474	KM101549	KM101511
M. americanum-1	Costa Rica	South/Central America-Pacific	CCDB 1731	HM352447	HM352489	KM101499
M. americanum-2	Río Aranjuez, Costa Rica	South/Central America-Pacific	MZUCR 3292-03	KM101473	KM101547	KM101504
M. americanum-3	Río Coronado, Costa Rica	South/Central America-Pacific	MZUCR 2963-01	KM101470	KM101544	KM101501
M. americanum-4	Río Oro, Costa Rica	South/Central America-Pacific	MZUCR 2964-01	KM101471	KM101545	KM101502
M. americanum-5	Isla Violines, Costa Rica	South/Central America-Pacific	MZUCR 2970-01	KM101472	KM101546	KM101503
M. americanum-6	Costa Rica	South/Central America-Pacific	CCDB 2883	JQ805797	JQ805899	JQ805843
M. americanum	Río Cabuya, Panama	South/Central America-Pacific	CCDB 2463	KM101469	KM101543	KM101500
M. heterochirus	Ilha de São Sebastião-SP, Brazil	South/Central America-Atlantic	CCDB 2137	HM352454	HM352494	KM101515
M. heterochirus-1	Río Suarez, Costa Rica	South/Central America-Atlantic	CCDB 2899	KM101477	KM101552	KM101516
M. heterochirus-2	Reserva Veragua, Costa Rica	South/Central America-Atlantic	CCDB 4875	KM101478	KM101553	KM101517
M. heterochirus	Veracruz, Mexico	South/Central America-Atlantic	Not available	KM101479	KM101554	KM101518
M. occidentale	Río Aranjuez, Costa Rica	North/Central America-Pacific	MZUCR 3292-02	KM101482	KM101557	KM101522
M. occidentale	Oaxaca, Mexico	North/Central America-Pacific	CNCR 24838	KM101481	KM101556	KM101521

Species	**Locality**	**Distribution**	**Catalogue Nº**	**16S**	**COI**	**18S**
Other palaemonids						
M. borellii	Buenos Aires, Argentina	South America-Inland waters	UFRGS 3669	HM352426	HM352480	KM101505
M. brasiliense	Serra Azul-SP, Brazil	South America-Inland waters	CCDB 2135	HM352429	HM352481	KM101506
M. jelskii	Pereira Barreto-SP, Brazil	South America-Inland waters	CCDB 2129	HM352437	HM352484	KM101519
M. michoacanus	Oaxaca, Mexico	Mexico-Inland waters	CNCR 24837	KM101480	KM101555	KM101520
M. potiuna	Eldorado-SP, Brazil	Brazil-Inland waters	CCDB 2131	HM352438	KM101564	KM101530
M. rosenbergii	Culture, Brazil	Indo-Pacific	CCDB 2139	HM352465	-	KM101531
M. rosenbergii	Kaohsiung Co., Taiwan	Indo-Pacific	Not informed	-	AB235295	-
M. surinamicum	Icangui-PA, Brazil	South America-Atlantic	INPA-CR 183	HM352446	KM101565	KM101532
Cryphiops caementarius	Region IV, Chile	South America-Pacific	CCDB 1870	HM352453	HM352495	KM101490
Palaemonetes argentinus	Parati-RJ, Brazil	South America	CCDB 2011	HM352425	-	KM101536
Palaemonetes argentinus	Not informed	South America	Not informed	-	HQ587179	-

Ribeirão Preto (FFCLRP), University of São Paulo (USP), National Institute of Research of Amazônia (INPA) – Brazil, and the Museum of Zoology, School of Biology, University of Costa Rica, Costa Rica (MZUCR). The collections of species conducted in this study complied with current applicable state and federal laws.

Additional material was obtained by donation, visit or loan from distinct worldwide crustacean collections (Table 1). A total of 65 specimens of *Macrobrachium* and three of other genera were analyzed. Almost all sequences were generated in the Laboratory of Bioecology and Crustacean Systematics (LBSC). Some additional comparative sequences were retrieved from GenBank (Table 1). The selection of the other *Macrobrachium* species and genera was based on the phylogeny of Pileggi and Mantelatto (2010), including closely related as well as more phylogenetically distant species. The species identification was based on diagnostic morphological features in accordance with the literature (Holthuis 1952, Villalobos 1969, Melo 2003, Pileggi and Mantelatto 2012).

DNA extraction, amplification and sequencing

The molecular analysis was based on partial fragments of the 16S rDNA, 18S nDNA and COI mtDNA genes, which have been effective in solving different levels of relationships among decapod species (Schubart et al. 2000, 2001a, b, Porter et al. 2005, Pileggi and Mantelatto 2010, Mantelatto et al. 2011, Vergamini et al. 2011, Carvalho et al. 2013, Rossi and Mantelatto 2013).

DNA extraction, amplification and sequencing protocols followed Pileggi and Mantelatto (2010). Total genomic DNA was extracted from the muscle tissue of walking legs, the chelipeds, or the abdomen. An approximately 530-bp region of the 16S rDNA gene, 560-bp region of the COI gene and 550-bp region of the nuclear 18S gene were amplified from diluted DNA by means of a polymerase chain reaction (PCR) in an Applied Biosystems Veriti 96 Well Thermal Cycler® (thermal cycles: initial denaturing for 5 min at 95 °C; annealing for 40 cycles: 45s at 95 °C, 45s at 48–50 °C, 1 min at 72 °C; final extension 3 min at 72 °C) with the following primers: 16Sar and 16Sbr (Palumbi et al. 1991) for 16S mitochondrial gene; COI-a and COI-f (Palumbi and Benzie 1991) for COI mitochondrial gene; 18Sai and 18Sb3.0 (Whiting et al. 1997) for 18S nuclear gene. PCR products were purified using Sure Clean (Bioline) and sequenced with the ABI Big Dye® Terminator Mix (Applied Biosystems, Carlsbad, CA) in an ABI Prism 3100 Genetic Analyzer® (Applied Biosystems automated sequencer) following Applied Biosystems protocols. All sequences were confirmed by sequencing both strands. A consensus sequence for the two strands was obtained using the computational program BIOEDIT 7.0.5 (Hall 2005). Apart from that, the consensus sequences were blasted on GenBank and compared with our previous sequences. Genetic vouchers generated were deposited in the CCDB under the accession numbers listed in Table 1 or returned with an appropriate label to the original collections.

Molecular analyses

Sequences were aligned using CLUSTAL W (Thompson et al. 1994) with interface to BIOEDIT with default parameters. Ambiguous alignment regions were removed. Genetic-distance analyzes for the partial sequences of the three markers (16S rDNA, COI mtDNA and 18S nDNA), over sequence pairs between and within groups were conducted in MEGA 5.2 using Kimura-2-parameter model (Tamura et al. 2011). Sequences were analyzed under the Akaike Information Criterion (AIC) (Posada and Buckley 2004) with the program JMODELTEST 2.1.3 (Darriba et al. 2012) to find the best substitution model. The maximum likelihood (ML) analysis was carried out using PAUP 4.0b10 (Swofford 2003). The consistency of topologies was measured by the bootstrap method (1000 replicates), and only confidence values > 50% were reported.

Results

Our phylogenetic analysis included 12 transisthmian American species of *Macrobrachium*, 7 from other American *Macrobrachium* species, and 2 from palaemonid-related groups. We generated 95 new sequences: 26 mitochondrial 16S sequences, 25 mitochondrial COI sequences, and 44 nuclear 18S sequences. The analysis of the 181 sequences from the three genes produced an alignment of 1.645 bp.

The optimal model for the concatenated data set was the TPM1uf model of sequence evolution (Kimura 1981) plus gamma distributed rate heterogeneity with a significant proportion of invariable sites (TPM1uf +I+G) with the following parameters: assumed nucleotide frequencies A = 0.3028, C = 0.2125, G = 0.1909, T = 0.2937; proportion of invariable sites I = 0.6020; the variable sites followed a gamma distribution, with shape parameter = 0.6700.

The topology obtained by maximum likelihood from concatenated genes (16S, 18S and COI) analyses confirmed that the transisthmian sibling species (*M. heterochirus* × *M. occidentale* – Sibling 1, *M. carcinus* × *M. americanum* – Sibling 2, *M. olfersii* × *M. digueti* – Sibling 3, *M. crenulatum* × *M. hancocki* – Sibling 4, and *M. acanthurus* × *M. tenellum* – Sibling 5) are closely related by well-supported clades (Fig. 1). Sibling 6 (*M. amazonicum* × *M. panamense*) did not form a separate sister clade despite being phylogenetically close. The position of *Palaemonetes argentinus* showed a stable condition in an external branch. However, the other outgroup (*Cryphiops caementarius*) was maintained within the *Macrobrachium* clade in the phylogeny (Fig. 1). The results did not reveal geographical separation among populations of the same species inside each group (Siblings 1–5). *Macrobrachium michoacanus* (see the arrow in the phylogeny) seems to be close related to *M. hancocki* in Sibling 4 group.

The relation among the sibling groups is supported by morphological traits. The species included in Siblings 1 and 2 exhibit similar shapes of the rostrum with the upper margin somewhat arched over the eye and with the apex directed upward (Fig. 1C). Species of the Siblings 3 and 4 with *M. michoacanus* and *M. surinamicum* show simi-

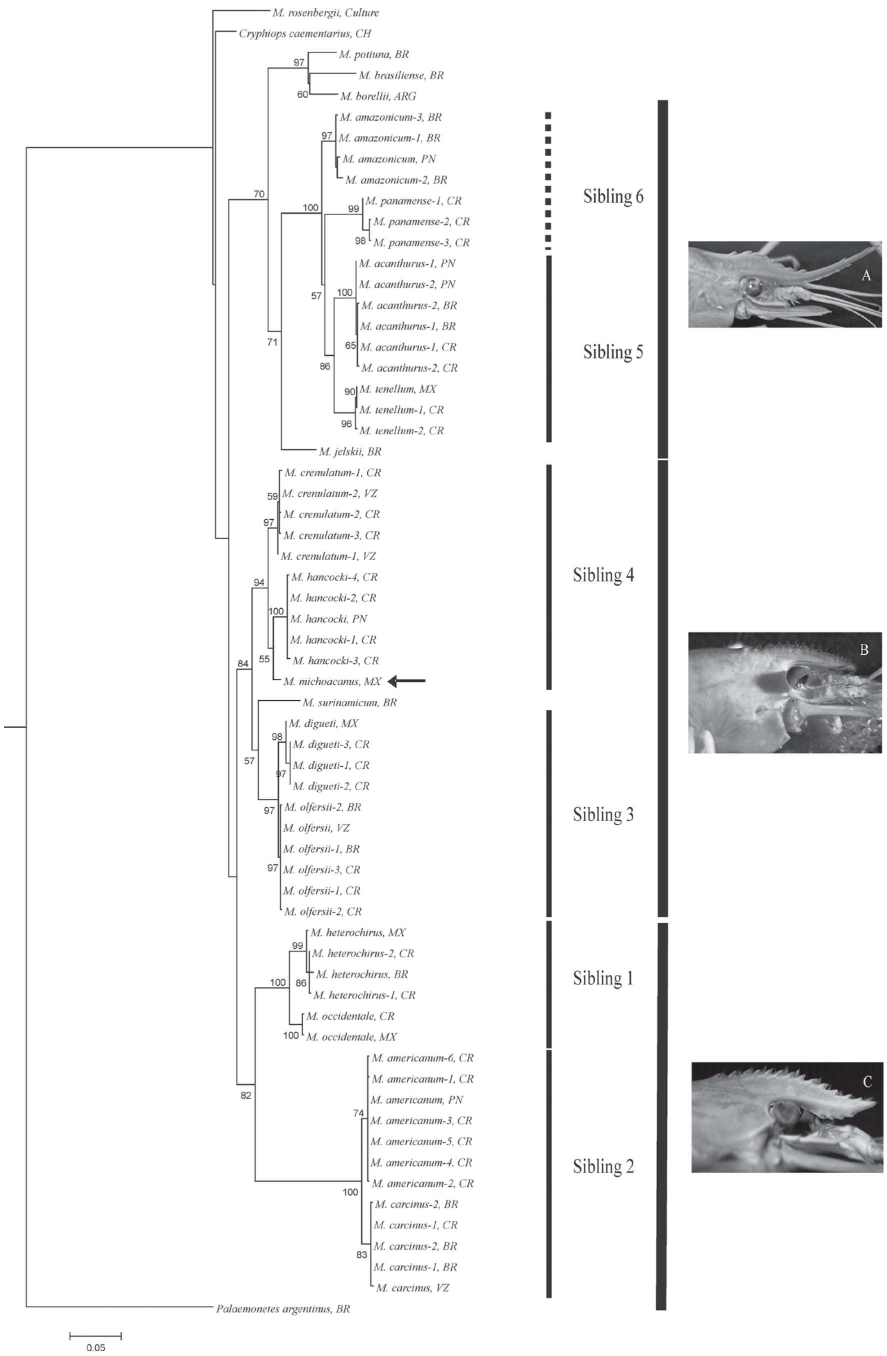

Figure 1. Phylogenetic tree obtained from concatenated maximum likelihood analysis of 16S, COI and 18S sequences for *Macrobrachium* sibling species. Numbers are significance values for 1000 bootstraps; values ≤ 50% are not shown. Abbreviations: ARG: Argentina; BR: Brazil; CH: Chile; CR: Costa Rica; MX: Mexico; PN: Panama; VZ: Venezuela. A: lateral view of the rostrum of *M. amazonicum*; B: lateral view of the rostrum of *M. olfersii*. C: lateral view of the rostrum of *M. carcinus*.

lar rostrum, being almost straight and usually with more than 10 teeth in the upper margin (Fig. 1B). In the same way the species of Siblings 5 and 6 and *M. jelskii* have a distinct rostrum, which is elongated, slender, with apex curved upward, with many teeth in the upper and lower margin (Fig. 1A).

In general, distance analyses revealed that the percentage of intraspecific variation was lower than interspecific variation (Table 2). Considering the relation between distinct sibling species, the genetic variability ranged from 4.4% (Sibling 3 × Sibling 4) to 16.9% (Sibling 4 × Sibling 6) for 16S, from 11.3% (Sibling 3 × Sibling 4) to 23.9% (Sibling 2 × Sibling 5) for COI, and from 1.1% (Sibling 5 × Sibling 6) to 11.3% (Sibling 2 × Sibling 5, 6) for 18S (Table 2). Inside each sibling group, the genetic variability varied between 1.5% (Sibling 3) and 8.7% (Sibling 6) for 16S, between 8.3% (Sibling 2) and 16.9% (Sibling 5) for COI, and between 0.0% (Sibling 2, 3) and 1.1% (Sibling 1) for 18S (Table 2).

Discussion

Over 150 sequences from three different gene regions were used in the present study in order to estimate phylogenetic relationships among freshwater prawns of the genus *Macrobrachium*, which previously were assumed to be transisthmian sibling species. The results revealed that all geminate species studied herein were valid taxonomic entities. Likewise they confirmed the role of the Isthmus of Panama as an effective barrier contributing in the separation of sibling species by the mechanism of allopatric speciation. However, in other cases the separation happened before the closure of the Isthmus probably by the mechanism of sympatric speciation. Our multigenic phylogeny produced consistent groups in most of the pairs of geminate species *i.e.*, sister taxa geographically separated: *Macrobrachium heterochirus* × *M. occidentale*, *M. carcinus* × *M. americanum*, *M. olfersii* × *M. digueti*, *M. crenulatum* × *M. hancocki* and *M. acanthurus* × *M. tenellum*. The constitution of these clades corroborates the morphological proximity of each pair of species as mentioned by Holthuis (1952).

The genetic divergence analyses showed the separation of each sibling group from others, which suggest a consistent relation in comparison with other congeners species (Table 2). Considering that for 16S the divergence in decapods is presumed to be around between 0.6 to 0.9% per Myr (Schubart et al. 2000), we can estimate the divergence time of the sibling species according to the closure of the Isthmus. For Siblings 1 and 5 the time of divergence between the species was approximately from 5.1 to 7.8 and 7.11 to 11.5 Mya, respectively. These estimates predate the closure of the Isthmus, which suggest that the speciation process separated these two species already before the closure. Considering that these amphidromous species are dependent of estuarine water for successful larval development, a sympatric speciation hypothesis seems to be unlikely. However, in these cases the possibility of occurrence of this event is plausible, probably due to environmental changes (Knowlton et al. 1993, Knowlton and Weigt 1998, Morrison et al. 2004). A genetic differentiation could have arisen from a mu-

Table 2. Genetic divergence matrix of the 16S and COI mitochondrial genes and 18S nuclear gene among American *Macrobrachium* sibling species obtained by distance analyses using Kimura-2-parameter model. SB: Sibling species. Comparison between the same sibling (bold numbers) comprises interspecific and intraspecific (numbers in parenthesis) analyses.

		SB1	SB2	SB3	SB4	SB5	SB6
16S	**SB1**	**0.047–0.046 (0.002–0.013)**					
	SB2	0.088–0.103	**0.019–0.028 (0.000–0.006)**				
	SB3	0.076–0.093	0.084–0.102	**0.015–0.019 (0.000–0.004)**			
	SB4	0.081–0.097	0.076–0.098	0.044–0.065	**0.017–0.021 (0.000–0.011)**		
	SB5	0.095–0.136	0.107–0.125	0.115–0.128	0.117–0.136	**0.064–0.069 (0.000–0.004)**	
	SB6	0.112–0.146	0.114–0.149	0.115–0.155	0.117–0.169	0.062–0.097	**0.075–0.087 (0.002–0.011)**
COI	**SB1**	**0.110–0.128 (0.011–0.061)**					
	SB2	0.175–0.233	**0.083–0.122 (0.000–0.038)**				
	SB3	0.149–0.179	0.159–0.204	**0.103–0.119 (0.004–0.022)**			
	SB4	0.136–0.179	0.168–0.205	0.113–0.168	**0.086–0.109 (0.006–0.091)**		
	SB5	0.156–0.197	0.167–0.239	0.147–0.191	0.168–0.209	**0.160–0.169 (0.000–0.022)**	
	SB6	0.151–0.180	0.161–0.234	0.143–0.190	0.148–0.196	0.138–0.187	**0.141–0.152 (0.004–0.040)**
18S	**SB1**	**0.011 (0.000)**					
	SB2	0.097–0.100	**0.000 (0.000)**				
	SB3	0.059–0.097	0.097	**0.000 (0.000)**			
	SB4	0.044–0.097	0.094–0.097	0.022–0.025	**0.008 (0.000)**		
	SB5	0.056–0.059	0.110–0.113	0.053–0.056	0.041–0.047	**0.003 (0.000)**	
	SB6	0.056–0.061	0.103–0.113	0.047–0.056	0.039–0.047	0.000–0.011	**0.008 (0.000)**

tational step and the two subpopulations, whose geographic distribution ranges overlap completely, became isolated because both occupy completely different ecological niches (Bush 1994). Analogous events were reported for other crustacean (Malay and Paulay 2009, Jarman et al. 2011). In addition, the estuary can contribute to restriction of the gene flow between the species by distinct selective regimes or habitat fidelity of the species, generating potential speciation in complete or partial isolation (Stanhope et al. 1992, Bilton et al. 2002). Therefore, the sympatric speciation may have occurred in these sibling species by the mechanism of microallopatry (Fitzpatrick et al. 2008). The difficulty in separating *M. heterochirus* from *M. occidentale*, and *M. acanthurus* from *M. tenellum* using morphological, ecological and genetic characters (Tables 2, 3, 5), allied

Table 3. Distributional and ecological comparison among each *Macrobrachium* species of sibling pair 1 and 2.

	Sibling 1		Sibling 2	
	M. occidentale	***M. heterochirus***	***M. americanum***	***M. carcinus***
American slope	Pacific	Atlantic	Pacific	Atlantic
Distribution	Mexico to Panama	USA (Florida) to Brazil (Rio Grande do Sul)	Mexico (Baja California) to Peru	USA (Florida) to Brazil (Rio Grande do Sul)
Habitat	wide range of altitudes (more common in higher elevations of the rivers)		wide range of altitudes (more common in medium and higher courses of the rivers	
Reproduction	require brackish water for reproduction (extended larval development with numerous and small eggs)		require brackish water for reproduction (extended larval development with numerous and small eggs)	
Morphology	very similar and just a few morphological details better seen in adult males are useful characters to separate both species		very similar and present few distinct morphological characters	
References	Holthuis 1952, Mejía-Ortiz et al. 2001, Rocha and Bueno 2004, Almeida et al. 2008, Lara 2009, Villalobos-Hiriart et al. 2010, Lara and Wehrtmann 2011, García-Guerrero et al. 2013, Pileggi et al. 2013		Holthuis 1952, Choudhury 1971, Monaco 1975, Bowles et al. 2000, Mejía-Ortíz et al. 2001, Hernández et al. 2007, Valencia and Campos 2007, Almeida et al. 2008, Lara 2009, Pileggi and Mantelatto 2010, Lara and Wehrtmann 2011, García-Guerrero et al. 2013	

Table 4. Distributional and ecological comparison among each *Macrobrachium* species of sibling pair 3 and 4.

	Sibling 3		Sibling 4	
	M. digueti	***M. olfersii***	***M. hancocki***	***M. crenulatum***
American slope	Pacific	Atlantic	Pacific	Atlantic
Distribution	Mexico (Baja California) to Ecuador	USA (Florida) to Brazil (Rio Grande do Sul)	Costa Rica to Ecuador	West Indies, Panama, Colombia and Venezuela
Habitat	wide range of altitudes (more common in higher elevations of the rivers)		wide range of altitudes (more common in higher elevations of the rivers)	
Ecology	require brackish water for reproduction (extended larval development with numerous and small eggs)		require brackish water for reproduction (extended larval development with numerous and small eggs)	
Morphology	very alike a few characters better seen in adult males are used to separate both species		very similar and can be differentiated using the color pattern, but fixed specimens are difficult to distinguish using only morphological characters	
References	Holthuis 1952, Dugger and Dobkin 1975, Abele and Kim 1989, Wicksten 1989, Rodríguez-Almaraz and Campos 1996, Mejía-Ortiz et al. 2001, Melo 2003, Valencia and Campos 2007, Almeida et al. 2008, Lara 2009, Mejía-Ortiz and Álvarez 2010, Lara and Wehrtmann 2011, Pileggi and Mantelatto 2012, Anger 2013, García-Guerrero et al. 2013, Rossi and Mantelatto 2013		Holthuis 1950, 1952, Wicksten 1989, March et al. 1998, Valencia and Campos 2007, Hein et al. 2011, Lara and Wehrtmann 2011, Anger 2013, García-Guerrero et al. 2013	

Table 5. Distributional and ecological comparison among each *Macrobrachium* species of sibling pair 5 and "6".

	Sibling 5		**"Sibling 6"**	
	M. tenellum	***M. acanthurus***	***M. panamense***	***M. amazonicum***
American slope	Pacific	Atlantic	Pacific	Atlantic
Distribution	Mexico (Baja California) to Peru	USA (North Caroline) to Brazil (Rio Grande do Sul)	Honduras to Peru	South American river basins from Venezuela to Argentina
Habitat	wide range of altitudes (more common in median courses of the rivers)		wide range of altitudes (more common in higher elevations of the rivers)	
Ecology	require brackish water for reproduction (extended larval development with numerous and small eggs)		require brackish water for reproduction (extended larval development with numerous and small eggs)	inland (independent of salty water to reproduction) and coastal populations (dependent of salty water to reproduction) (distinct forms of extended larval development with numerous and small eggs)
Morphology	similar and difficult to distinguish		similar, and only few characters are useful features to separate both species	
References	Holthuis 1952, Choudhury 1970, Melo 2003, Hernández et al. 2007, Almeida et al. 2008, Lara and Wehrtmann 2011, Anger 2013, García-Guerrero et al. 2013.		Holthuis 1952, Abele and Kim 1989, Melo 2003, Magalhães et al. 2005, Valencia and Campos 2007, Almeida et al. 2008, Lara 2009, Jara 2010, Vergamini et al. 2011, Anger 2013, Meireles et al. 2013	

with the consistent position in the phylogeny (Fig. 1) provide convincing arguments to consider them as sibling species. The phylogenetic position of Siblings 1 with 2 and Siblings 5 with “6” (here marked between quotes due its artificial position, not characterized as sibling) followed the morphological grouping based on the shape of the rostrum (Fig. 1A, C) indicating that this character is determinant for taxonomic studies.

The time of divergence between both species of the Sibling 3 was approximately from 1.66 to 3.16 Mya for 16S gene, which supports the efficiency of the barrier in the separation of sibling species by mechanism of allopatric speciation. The morphologically close relation of the “olfersii complex” (see Villalobos 1969 for revision) was corroborated in the phylogeny, where Siblings 3 and 4 form sister groups with the same shape of the rostrum (Fig. 1B), as evidenced in previous molecular results (Rossi and Mantelatto 2013). The entity of the results obtained together with morphological and ecological similarities of *M. olfersii* and *M. digueti* suggest that both are sibling species, but the inclusion of other species from the *M. olfersii* complex in the analysis is necessary to confirm this proposition. Among the sibling species proposed by Holthuis (1952), only one pair (*M. surinamicum* × *M. transandicum*) was not analyzed in our study due the impossibility to obtain specimens of *M. transandicum*. In our phylogeny, *M. surinamicum* was included inside the clade of *Macrobrachium olfersii* complex (Villalobos 1969, Rossi and Mantelatto 2013) corresponding to a species with the rostrum almost straight, usually with more than 10 teeth in the upper margin (Fig. 1B).

For Siblings 2 and 4 the time of divergence between the species varied from 2.11 to 4.66 and 1.88 to 3.5 Mya for 16S gene, respectively. These data place them exactly in the range of the closure of the Isthmus, precluding the definition that the separation of the species may have been caused by this vicariant process. Pileggi and Mantelatto (2010) mentioned that *M. americanum* could be a synonymous of *M. carcinus* based on a single molecular 16S phylogeny. However, and as suggested by the authors, a more extensive sampling of *M. americanum* will be necessary to verify this proposition. Our results that include five specimens of *M. carcinus* and seven of *M. americanum* from distinct localities revealed that both species are sibling species.

In the same way, our data as well as the morphological and ecological similarities evidenced the close relationship between *M. crenulatum* and *M. hancocki*; however, the addition of data from more specimens and other species from the *M. olfersii* complex is necessary to confirm them as sibling species, *i.e.*, sister taxa geographically separated (Rossi and Mantelatto, unpubl. data). Another unpredictable result was the close relation of *M. michoacanus* with *M. hancocki* (Fig. 1, Sibling 4). With both occurring on the Pacific side, this result may be interpreted as an indication that the relation of phylogenetically closely related congeners living on either side of the Isthmus must be older than the biogeographic barrier separating them (Anger 2013). New diversifications succeeding the closure of the Isthmus occurred at the same side, which can be demonstrated by the higher proximity between these sympatric species than *M. hancocki* and *M. crenulatum*, the hypothetical Sibling 4. However, analysis of additional material is necessary to verify the phylogenetic position of *M. michoacanus*.

Following the other sibling species, relationship of the systematic position with the shape of the rostrum was maintained (Fig. 1B–C) supporting the high reliability of this morphological character.

Our results regarding *M. amazonicum* × *M. panamense* did not confirm a separate sibling group despite the close phylogenetic relation among these species. Our multigenic phylogenetic hypothesis (Fig. 1) indicates *M. panamense* as a sister group of the Sibling 5, and *M. amazonicum* as a sister species of this group (*M. panamense* + Sibling 5). Genetic divergence analyses of the "Sibling 6" pair (8.33 to 14.5 Mya for 16S genes) suggest that the time of their divergence predates the closure of the Isthmus, indicating that both did not share the same ancestral. In addition, the wide geographic distribution of *M. amazonicum* in the large South American river basins must be related to geological events driven by the rising Andes along the western portion of these basins (supposedly its native area of occurrence) (see Magalhães et al. 2005 for revision). *Macrobrachium jelskii* as an external clade of Sibling 5 and "Sibling 6" is in agreement with morphological similarities among these species, mainly of the shape of the rostrum (Fig. 1A), despite *M. jelskii* being the unique species of the group to present abbreviated larval development. The position of a more external group (*M. potiuna*, *M. brasiliense*, *M. borellii*) with abbreviated larval development in the phylogeny indicates that the ancestral species of this entire group possibly had a life cycle independent of salt water as suggested in previously studies (Murphy and Austin 2005, Pileggi and Mantelatto 2010). *Macrobrachium amazonicum* plays a key role in this puzzle since it presents inland and coastal populations (Vergamini et al. 2011, Meireles et al. 2013), suggesting that the species originated in freshwater environments and entered subsequently in estuarine habitats (Pereira and García 1995, Pileggi and Mantelatto 2010).

Phylogenetic analyses were based on two mitochondrial and one nuclear genes in order to provide a broad spectrum of inference and insights into the evolutionary history of *Macrobrachium* in the Americas. Although the mitochondrial markers may offer strong evidence for genus and species-level relationships, they have high mutation rates, which can cause increasing saturation when older splits are analyzed (Simon et al. 1994, Avise 2004). Therefore, analyses were carried out with sequences from conserved and variable genes to access phylogenetic information across a range of evolutionary time (Crandall et al. 2000). The genes concatenated analysis improve the diversity of evolutionary time, consequently revealed a more consistent phylogeny compared to previous morphological and molecular phylogenetics studies (Pereira 1997, Murphy and Austin 2005, Pileggi and Mantelatto 2010). The inclusion of the member of genus *Cryphiops* within *Macrobrachium* species was maintained in the phylogeny, and raises the question whether *Macrobrachium* is a monophyletic group (Pereira 1997, Murphy and Austin 2005, Pileggi and Mantelatto 2010, Carvalho et al. 2013, Rossi and Mantelatto 2013).

The results of our multidisciplinary approach suggest that species pairs 1-5 refer to siblings, in which each pair of species is difficult to distinguish using traditional morphological characters, although they are genetically distinct, closely related, and reproductively isolated (Mayr 1963, Steyskal 1972, Knowlton 1986). In contrast, our data did not validate "Sibling 6" by molecular analysis, although morphology, ecology, and geographic distribution patterns seem to suggest that they are sibling species (Holthuis 1952). Moreover, the speciation processes of the species of the pairs 2, 3, and 4 seem to have occurred after the rise of the isthmus barrier, probably in Pliocene and Pleistocene by the mechanism of allopatric speciation. However, the isolation of pairs 1 and 5 may have happened before the rise of the isthmus barrier, probably in Miocene by the mechanism of sympatric speciation.

An intriguing case refers to the occurrence of two species (*M. hobbsi* and *M. olfersii*) on both sides of the Central American land bridge (Anger 2013). The identification of these species may be incorrect or is related to the possibility of a passageway that has started with the opening of the Panama Canal, a scenario that has been already reported (Hildebrand 1939, Abele and Kim 1989). The possible expansion of the distribution range of *Macrobrachium* through the Panama Canal may occur, especially considering the dispersal potential of these amphidromous species (Bauer and Delahoussaye 2008, Bauer 2011, 2013). However the findings of the previous genetic study with *M. olfersii* revealed the absence of gene flow between Pacific and Atlantic populations. Moreover, *M. digueti* and specimens from Pacific considered as *M. olfersii* did not show divergence enough to split them, and the differences were within the range of interspecific values. Therefore, on the Pacific coast only *M. digueti* occurs naturally, which is considered, like *M. olfersii*, a sibling and cryptic species (Rossi and Mantelatto 2013).

This is the first phylogenetic study using molecular methods devoted entirely to the American transisthmian *Macrobrachium* sister species. Molecular markers confirmed that the Isthmus of Panama is an effective barrier contributing to the separation of sibling freshwater prawns species by the mechanism of allopatric speciation. However, some species seemed to have evolved before the closure of the Isthmus by the mechanism of sympatric speciation. Our phylogenetic analysis revealed consistent groups in most of the studied pairs endorsing the supposed sibling species. In contrast, the position of one pair (*M. amazonicum* × *M. panamense*) seems to be artificial once they did not share a recent common ancestor. The results presented here contribute to resolution of some doubts about the relationships of geminate American species. Our results support the conclusion that these sibling species are valid taxonomic entities, but not all transisthmian species are the closest living relatives with each other.

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Results of the global conservation assessment of the freshwater crabs (Brachyura, Pseudothelphusidae and Trichodactylidae): The Neotropical region, with an update on diversity

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Abstract

The freshwater crabs of the Neotropics comprise 311 species in two families (Pseudothelphusidae and Trichodactylidae) and one or both of these families are found in all of the countries in the Neotropical region (except for Chile and some of the Caribbean islands). Colombia (102 species, 81% endemic) and Mexico (67 species, 95% endemic) are the biodiversity hotspots of freshwater crab species richness and country-level endemism for this region. The results of the IUCN Red List conservation assessments show that 34% of pseudothelphusids and 10% of trichodactylids have an elevated risk of extinction, 29% of pseudothelphusids and 75% of trichodactylids are not at-risk (Least Concern), and although none are actually extinct, 56% of pseudothelphusids and 17% of trichodactylids are too poorly known to assess (Data Deficient). Colombia (14 species), Venezuela (7 species), Mexico (6 species), and Ecuador (5 species) are the countries with the highest number of threatened species of Neotropical freshwater crabs. The majority of threatened species are restricted-range semiterrestrial endemics living in habitats subjected to deforestation, alteration of drainage patterns, and pollution. This underlines the need to prioritize and develop conservation measures before species decline to levels from which they cannot recover. These results represent a baseline that can be used to design strategies to save threatened Neotropical species of freshwater crabs.

Keywords

Pseudothelphusidae, Trichodactylidae, Neotropical region, conservation, distribution, endemism

Introduction

The Neotropical region occupies the entire South American continent, plus Mexico and Central America, the islands of the Caribbean, and southern Florida. The Nearctic/Neotropical boundary runs through Mexico and passes through the southern parts of Baja California and Sonora, crosses the Meseta Central, and continues to southern Veracruz. The majority of the Neotropical region has a tropical climate with warm water freshwater ecosystems, but the southern part of this region from 10° to 25°S (southern Brazil, Uruguay, Paraguay, Argentina, and Chile) has a subtropical climate with cooler freshwater habitats. Freshwater crabs are found throughout the freshwater ecosystems of the Neotropical region but are notably absent from Baja California and the Yucatan Peninsula (Mexico), southern Florida (USA), some of the Caribbean islands, southern Argentina, and all of Chile.

Our knowledge of the impressive freshwater crab fauna in this region has been very slow to develop since the first species of trichodactylid and pseudothelphusid freshwater crabs were described in 1783 and 1840, respectively. Only one species (a trichodactylid) was described in the 18th C (Herbst 1783), 46 more species were described in the 19th C (Ortmann 1893, 1897; Rathbun 1893, 1898), 209 more species were added in the 20th C (Rathbun 1905, Smalley 1964a,b, 1965, 1970, Bott 1967, 1968, 1969, 1970, Rodríguez and Smalley 1969, Pretzmann 1972, 1975, Hobbs 1980, Rodríguez 1980, 1982, Campos and Rodríguez 1984, 1985, 1993, 1995, Smalley and Adkison 1984, Magalhães 1986, Magalhães and Türkay 1986, Rodríguez 1986, Alvarez 1987, 1989, Campos and Rodríguez 1988, Campos 1989, Alvarez and Villalobos 1990, 1991, 1994, 1995, 1996, 1997a,b, 1998, Hobbs 1991, Campos 1992, 1999, Rodríguez 1992, Villalobos et al. 1993, Campos 1995, 1997, Alvarez et al. 1996, 1999, Magalhães and Türkay 1996a–c, Campos and Lemaitre 1998), and 31 species have been described so far in the 21st C (Campos 2000, 2001, 2002, 2003a, b, 2004, 2005, 2010a, b, 2011, Campos and Lemaitre 2002, Rodríguez et al. 2002, Villalobos and Alvarez 2003, 2009, 2010, 2013, Campos and Valencia 2004, Rodríguez and Magalhães 2005, Villalobos 2005, Campos and Pedraza 2006, 2008, Cumberlidge 2007, Magalhães and Türkay 2008a, b, c, 2012, Ng et al. 2008, Yeo et al. 2008, Cumberlidge et al. 2009, Magalhães 2010, Magalhães and Türkay 2010, Magalhães et al. 2010, Villalobos et al. 2010, Alvarez et al. 2012a, Cardona and Campos 2012, Magalhães et al. 2013). By 2009, the total number of Neotropical species stood at more than 300, making this the second most species-rich region in the world after the Oriental region (Ng et al. 2008, Cumberlidge et al. 2009). Interest in the Neotropical freshwater crab fauna remains strong and 13 new species have already been recognized since 2009 (Table 1). Exploration is still continuing and there is every prospect that the species count will increase further as taxonomic discrimination improves. We expect most of

Table 1. Species of Neotropical freshwater crabs described since Cumberlidge et al. (2009).

Family	Subfamily/Tribe	Species	Country
Pseudothelphusidae	Kingsleyini	*Microthelphusa lipkei* Magalhães, 2010	Brazil
Pseudothelphusidae	Kingsleyini	*Brasiliothelphusa dardanelosensis* Magalhães & Türkay, 2010	Brazil
Pseudothelphusidae	Strengerianini	*Phallangothelphusa juansei* Campos, 2010	Colombia
Pseudothelphusidae	Strengerianini	*Phallangothelphusa martensis* Cardona & Campos, 2012	Colombia
Pseudothelphusidae	Hypolobocerini	*Neostrengeria alexae* Campos, 2010	Colombia
Pseudothelphusidae	Hypolobocerini	*Neostrengeria natashae* Campos, 2011	Colombia
Pseudothelphusidae	Potamocarcinini	*Potamocarcinus darienensis* Magalhães, Campos & Türkay, 2013	Panama
Pseudothelphusidae	Hypolobocerini	*Allacanthos yawi* Magalhães, Lara & Wehrtmann, 2010	Costa Rica
Pseudothelphusidae	Potamocarcinini	*Odontothelphusa apicpac* Villalobos, García & Velázquez, 2010	Mexico
Pseudothelphusidae	Potamocarcinini	*Sylvathelphusa kalebi* Villalobos & Alvarez, 2013	Mexico
Pseudothelphusidae	Potamocarcinini	*Sylvathelphusa cavernicola* Villalobos & Alvarez, 2013	Mexico
Pseudothelphusidae	Potamocarcinini	*Tzotzithelphusa villarosalensis* Villalobos & Alvarez, 2013	Mexico
Pseudothelphusidae	Pseudothelphusini	*Pseudothelphusa zongolicae* Alvarez, Villalobos & Moreno, 2012	Mexico

the new species to belong to the Pseudothelphusidae, because discovery of new species of trichodactylids has slowed dramatically in the last two decades.

Research on the Neotropical freshwater crabs has also focused on their phylogeny (Rodríguez and Campos 1989, 1998, Rodríguez and Pereira 1992, Rodríguez and Sternberg 1998, Sternberg et al. 1999, Sternberg and Cumberlidge 2001, Rodríguez and Magalhães 2005, Villalobos and Alvarez 2010), their biogeography (Rodríguez 1986, Campos 2005, Huidobro et al. 2006, Alvarez et al. 2012b, Ojeda et al. 2013), and their medical importance with 22 species of pseudothelphusids serving as second intermediate hosts of the human lung fluke, *Paragonimus* (Acha and Szyfres 2001) in 9 countries from Mexico and Central America, to the Andes, and Brazil (Rodríguez and Magalhães 2005).

The objectives of the present study are to describe and update our knowledge of Neotropical freshwater crab diversity of both the Pseudothelphusidae and Trichodactylidae and to describe the patterns of distribution and endemism within these families. We also identify here not only those species that are most vulnerable to extinction, but also those species that are poorly known and obvious candidates for future research attention.

Methods

Comprehensive distributional data for the Neotropical freshwater crabs were compiled from literature and museum records, particularly the major monographs on the Pseudothelphusidae by Rodríguez (1982, 1992) and Rodríguez and Magalhães (2005), and the Trichodactylidae by Rodríguez (1992) and Magalhães and Türkay (1996a–c, 2008a–b, 2012). Other regional monographs referred to include those for Colombia (Campos 2005), Ecuador (Rodríguez and Sternberg 1998), Brazil (Magalhães 2003), Peru (Rodríguez and Suárez 2004), and Mexico and Central America (Alvarez and Villalobos 1990, 1991, 1994, 1995, 1996, 1997, Villalobos et al. 1993, Alvarez et al. 1996, 1999, Villalobos and Alvarez 2003, 2008, 2009, 2010, Villalobos 2005). Despite these efforts distributional records for most species of freshwater crabs are still likely to be incomplete. Distribution maps presented here are based on specimen-level databases compiled for all species based on all known specimens and includes information from over 2,500 different localities. Individual conservation assessments for all species of Neotropical freshwater crabs assessed by Cumberlidge et al. (2009) using the IUCN Red List categories and criteria at the global scale (IUCN 2003) are provided on the IUCN Red List site (http://www.iucn.redlist.org). Freshwater crab species were assessed for inclusion in one of the Red List categories based on a combination of data on geographic range and/or population levels and related trends (Cumberlidge et al. 2009). The available data were sufficient to make valid assessments of the conservation status of only 150 out of the 298 species known from the Neotropical region at the time, and assessments were not possible for 148 species that were treated as Data Deficient due to a lack of specimens, and of locality and population data (Cumberlidge et al. 2009). Threats were inferred if a species was potentially subject to anthropogenic impacts such as habitat destruction, alteration of drainage patterns, or pollution, especially if it was either not found in a protected area, or if it was found in a protected area for only part of its range. Distribution maps (Figs 1–4) were prepared using ArcView GIS mapping software.

Results

Neotropical freshwater crab families

The Neotropical region has its own distinctly recognizable freshwater crab fauna, with no species occurring in other parts of the world. The Neotropical freshwater crab fauna is not uniformly distributed and species and genus composition changes from country to country (Table 2, Figs 1, 2). The global distributions of all species of the Pseudothelphusidae and Trichodactylidae based on all known localities are shown in figures 1 and 2. The Neotropical freshwater crab fauna today (49 genera, 311 species, 2 families) easily exceeds that of the entire Afrotropical region (18 genera, 145 species, 2 families), but is significantly less diverse than the Palaearctic-Oriental-Australasian region (147 genera, 850 species, 2 families) (Cumberlidge et al. 2009; present work).

The Neotropical freshwater crab fauna, which represent 20–22% of the world's freshwater crab species diversity (Ng et al. 2008, Yeo et al. 2008, Cumberlidge et al. 2009), is dominated by the highly diverse pseudothelphusids (34 genera, 264 species). Pseudothelphusids are found in 26 out of the 31 countries and reach their highest species richness in South America (Table 2). Pseudothelphusids are found throughout

Table 2. Distribution of Pseudothelphusidae (P) and Trichodactylidae (T) in each country in the Neotropical region that has populations of freshwater crabs. First number indicates the number of genera, species, and localities of Pseudothelphusidae, the second number indicates the same data for the Trichodactylidae.

	No. GENERA (P, T)	No. SPECIES (P, T)
South America		
Argentina	0, 5	0, 9
Bolivia	0, 6	0, 8
Brazil	6, 10	19, 31
Colombia	13, 8	88, 14
Ecuador	3, 4	21, 5
French Guiana	2, 4	3, 4
Guyana	3, 3	8, 3
Paraguay	0, 6	0, 7
Peru	2, 9	8, 14
Suriname	3, 3	5, 3
Uruguay	0, 1	0, 2
Venezuela	11, 7	35, 9
Mexico and Central America		
Mexico	15, 2	63, 4
Belize	4, 0	4, 0
Costa Rica	4, 0	14, 0
El Salvador	4, 0	6, 0
Guatemala	6, 0	12, 0
Honduras	2, 0	3, 0
Nicaragua	3, 3	5, 3
Panama	4, 1	14, 1
Caribbean		
Cuba	2, 0	9, 0
Dominica	1, 0	1, 0
Dominican Republic	1, 0	2, 0
Guadeloupe	1, 0	1, 0
Haiti	1, 0	1, 0
Martinique	1, 0	1, 0
Puerto Rico	1, 0	1, 0
Saint Vincent & the Grenadines	1, 0	1, 0
St Croix US Virgin Islands	1, 0	1, 0
Trinidad & Tobago	2, 1	2, 1

the warmer parts of the Neotropics from Mexico to Central America and the Andes (Venezuela, Colombia, Ecuador, and Peru), and in the Guyanian Shield and Central Brazilian Shield in the Amazon basin (Fig. 1, Table 2). The majority of pseudothelphusid species live at altitudes greater than 400 m above sea level, with a few Andean species reaching up to 3000 m (Campos 2005).

This family comprises two subfamilies, Epilobocerinae and Pseudothelphusinae (Ng et al. 2008). The Epilobocerinae consists of only 9 species in 2 genera (*Epilobocera*,

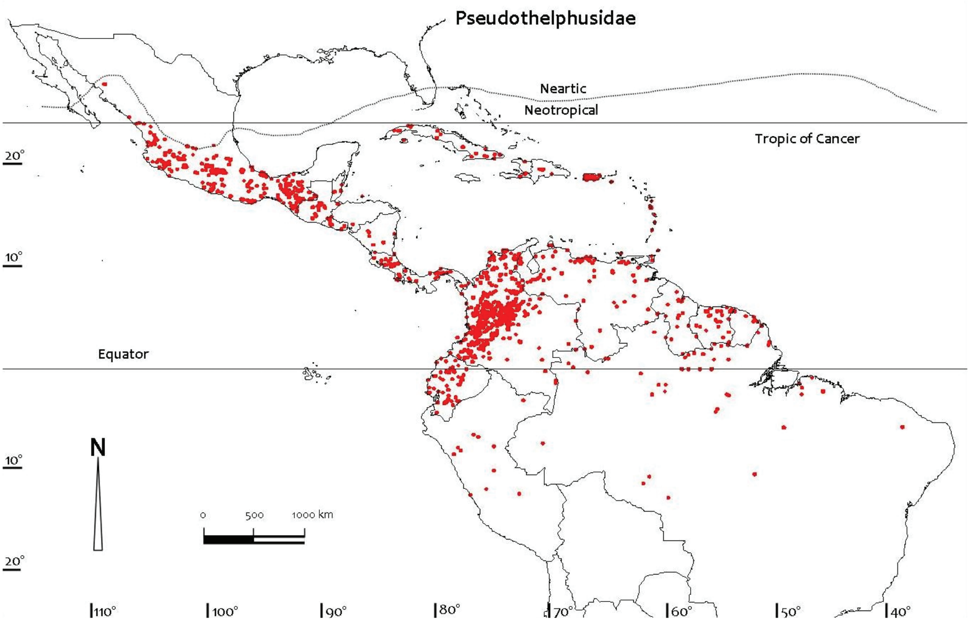

Figure 1. Distribution of Pseudothelphusidae based on all known point localities (n = 1719).

Neoepilobocera) that are all endemic to the larger islands of the Greater Antilles (Cuba, Haiti, Dominican Republic, Puerto Rico, and St. Croix). The vast majority of pseudothelphusids are in the subfamily Pseudothelphusinae assigned to five Tribes: Pseudothelphusini (Mexico), Potamocarcinini (Central America and Mexico), Hypolobocerini (from Venezuela to Peru, plus Central America, and Mexico), Strengerianini (Colombia and Venezuela), and Kingsleyini (the Lesser Antilles, Venezuela, Colombia, and northern Brazil). The eastern limits of the Pseudothelphusidae in the Amazon basin is in Ceará State, Brazil, while their southwestern limit in the Amazon basin is marked by the watershed of the Ucayali River, south of which there are no species of pseudothelphusids (Rodríguez and Magalhães 2005).

The Trichodactylidae is the smallest of all freshwater crab families (15 genera, 47 species) and represents only 4% of the world's freshwater crab diversity. Despite this, the family is widely distributed and has representatives in 16 countries, most of which are in South America (Table 2) (Rodríguez 1992, Ng et al. 2008, Yeo et al. 2008, Cumberlidge et al. 2009). The vast majority of trichodactylid species are found below 100 m above sea level in the Magdalena river basin and Lake Maracaibo in Colombia and Venezuela, in the coastal lowlands of the Guyanas and Brazil, and in the lowland river basins of the Amazon, Orinoco, Paraguay, and Parana. A few species of trichodactylids reach up to 900 m above sea level in the Andes foothills (Campos 2005). Countries with trichodactylids include Colombia, Venezuela, French Guiana, Guyana, Suriname, Brazil, Ecuador, Peru, Bolivia, Paraguay, Uruguay, and Argentina (Fig. 2, Table 2). Outside of South America there are four trichodactylid species in Veracruz, Tabasco, Oaxaca, and Chiapas in Mexico

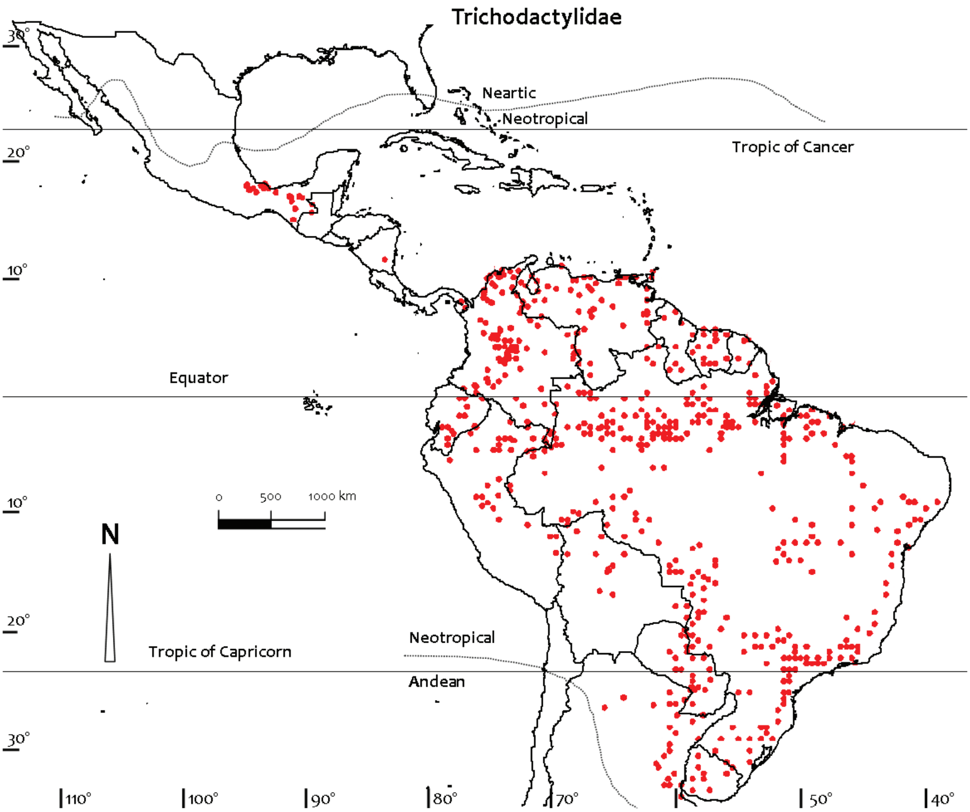

Figure 2. Distribution of Trichodactylidae based on all known point localities (n = 853).

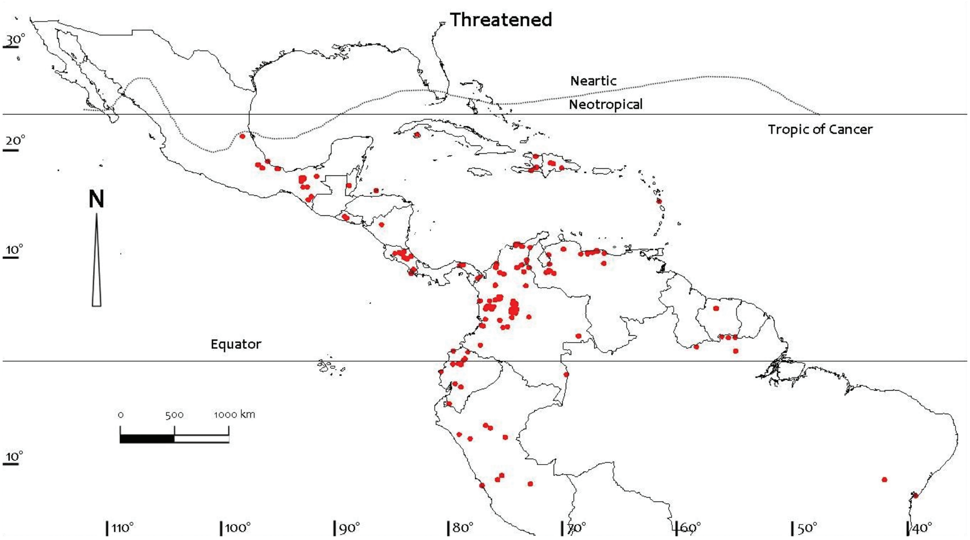

Figure 3. Distribution of threatened species of Neotropical freshwater crabs (Pseudothelphusidae and Trichodactylidae) based on all known point localities (n = 173).

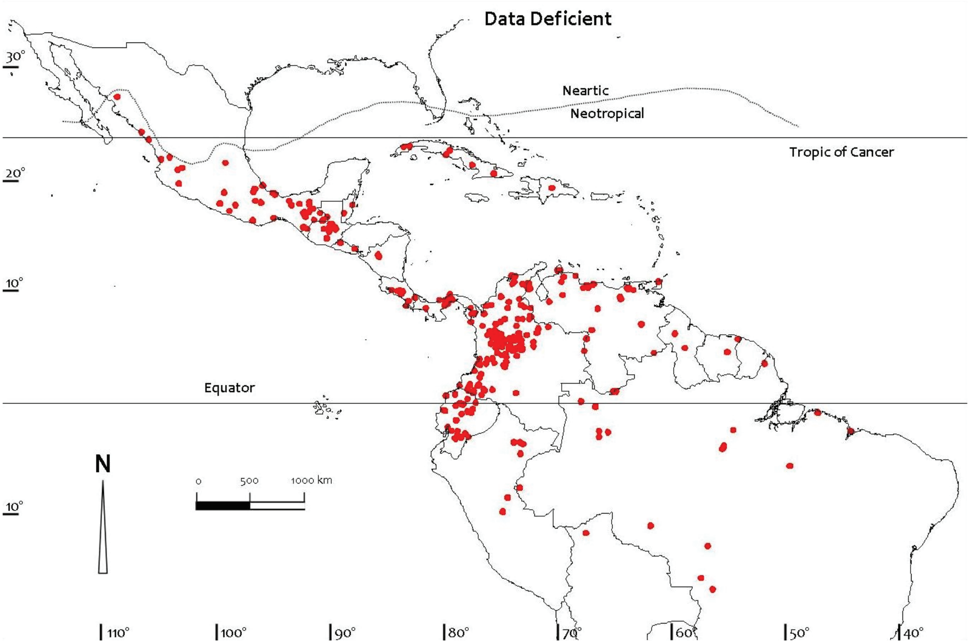

Figure 4. Distribution of Data Deficient species of Neotropical freshwater crabs (Pseudothelphusidae and Trichodactylidae) based on all known point localities (n = 338).

(*Avotrichodactylus constrictus*, *A. oaxensis*, *Rodriguezia mensabak*, and *R. villalobosi*) (Magalhães and Türkay 2012); one species (*Trichodactylus quinquedentatus*) from Colombia and Nicaragua (Smalley and Rodríguez 1972), and one species (*Poppiana dentata*) from Trinidad (which is also found on the neighboring mainland in Venezuela, Suriname, Guyana, and French Guiana, and in Nicaragua) (Magalhães and Türkay 2008a). The Trichodactylidae includes two subfamilies: the Trichodactylinae, with 3 genera, two from Mexico (*Avotrichodactylus* and *Rodriguezia*) and one from South and Central America (*Trichodactylus*); and the Dilocarcininae, with 9 genera that are found throughout South America (Rodríguez 1992, Magalhães and Türkay 1996a, b, c, 2008a, b). Interestingly, this classification (Ng et al. 2008) groups together at the subfamily level the four disjunct species of trichodactylids found in Mexico with the genus *Trichodactylus* that includes species from the Magdalena basin and Amazon slopes in Colombia, a species from Nicaragua and Colombia, species from the Atlantic coastal river systems of southeastern Brazil, and species from the Paraguay and Parana basins in southern Brazil and Argentina.

Hotspots of freshwater crab diversity

Colombia stands out as the most diverse and the most species rich part of the region (21 genera, 102 species, 2 families) (Table 2) with biodiversity hotspots located in forested mountainous regions where deep valleys and a complex topographical relief are favorable for genetic isolation and allopatric speciation. The pseudothelphusid fauna of Colombia comprises 13 genera and 88 species in one subfamily (Pseudothelphusinae) and four tribes (Hypolobocerini, Strengerianini, Kingsleyini, and Potamocarcinini). There are distinct northern and southern components of pseudothelphusid distribution in Colombia, with the dividing line coinciding with the watersheds of the San Juan, Cauca, and Magdalena river basins. The southern component of this fauna is dominated by *Hypolobocera*, *Lindacatalina*, and *Moritschus* and extends from southern Colombia into Ecuador and Peru, and also includes species from southeast Colombia and the Amazonian and Pacific slopes of Ecuador. The trichodactylid fauna of Colombia comprises 8 genera, 14 species in two subfamilies: Trichodactylinae (one genus) and Dilocarcininae (7 genera).

The next most species-rich country is Mexico (17 genera, 67 species, 2 families) (Table 2) with a freshwater crab fauna dominated by pseudothelphusids with 15 genera, and 63 species in one subfamily (Pseudothelphusinae) and three tribes (Pseudothelphusini, Potamocarcinini, and Hypolobocerini). Mexico's trichodactylid fauna is much smaller and comprises just 4 species in 2 genera all in the subfamily Trichodactylinae. Freshwater crab diversity in Mexico is highest in the Isthmus of Tehuantepec and Chiapas State, possibly because this is where species from three pseudothelphusid tribes overlap with species of trichodactylids (Villalobos and Alvarez 2010).

Brazil is the third most species-rich Neotropical country (16 genera, 50 species, 2 families) with a fauna that is dominated by trichodactylids (10 genera, 31 species, 2 subfamilies), and fewer pseudothelphusids (6 genera, 19 species, all Pseudothelphusinae, Kingsleyini). Venezuela is the fourth most species-rich Neotropical country (18 genera, 44 species, 2 families), made up of 11 genera and 35 species of pseudothelphusids (all Pseudothelphusinae, in three tribes, Hypolobocerini, Kingsleyini, and Strengerianini), and 7 genera and 9 species of trichodactylids (all Dilocarcininae).

The freshwater crab fauna of Ecuador (7 genera, 26 species, 2 families) includes 3 genera and 21 species of pseudothelphusids (all Pseudothelphusinae, Hypolobocerini), and 4 genera and 5 species of trichodactylids (all Dilocarcininae). Finally, Peru (11 genera, 22 species, 2 families) has a fauna dominated by trichodactylids (14 species and 8 genera, in 2 subfamilies Dilocarcininae and Trichodactylinae), with a smaller pseudothelphusid fauna consisting of 8 species and 2 genera (all Pseudothelphusinae in 2 tribes Kingsleyini and Hypolobocerini). Freshwater crabs of both families are completely absent from Chile. Despite warm subtropical climates freshwater crabs are absent in many islands of the Caribbean including Jamaica, The Cayman Islands, The Bahamas, Grenada, Antigua, and Anguilla.

When distribution patterns are considered at the genus level (Table 2) the taxonomic diversity of Neotropical freshwater crabs is again by far the highest in Colombia (21 genera), Venezuela (18 genera), Mexico (17 genera), Brazil (16 genera), and Peru (11 genera). Diversity is lower in Ecuador (7 genera), Guyana, Suriname, Bolivia, Paraguay, French Guiana, Nicaragua, and Guatemala (6 genera), and in Argentina and Panama (5 genera) (Table 2). Costa Rica, El Salvador and Belize each have 4 genera, Trinidad and Tobago has 3 genera, Honduras and Cuba each have 2 genera, while Uruguay and the other islands of the Greater and Lesser Antilles each have 1 genus.

Widespread species: Pseudothelphusidae

Thirteen (out of 34) pseudothelphusid freshwater crab genera and 28 (out of 283) species have a wide distribution that easily exceeds 20,000 km^2 and includes more than one country. Notably, *Fredius reflexifrons* is found in six countries (Brazil, French Guiana, Guyana, Peru, Suriname, Venezuela), *Potamocarcinus magnus* is found in five countries (Mexico, Guatemala, Honduras, El Salvador, Costa Rica), and *Kingsleya latifrons* (Guyana, French Guiana, Suriname, Brazil) and *Raddaus bocourti* (Mexico, Belize, Guatemala, El Salvador) are found in four countries. Other widespread species found in three countries include *Potamocarcinus armatus* (Mexico, Guatemala, Nicaragua), *P. richmondi* (Nicaragua, Costa Rica, Panama), *Fredius denticulatus* (Brazil, French Guiana, Suriname) and *F. fittkaui* (Venezuela, Guyana, Brazil), and *Prionothelphusa eliasi* (Brazil, Colombia and Venezuela). Finally, 17 widespread species are found in two countries: *Rodriguezus garmani* (Trinidad and Tobago, Venezuela), *Fredius beccarii* (Guyana, Venezuela), *Zilchia zilchi* (El Salvador, Honduras), *Hypolobocera caputii* and *Moritschus henrici* (both Ecuador, Peru), *Ptychophallus exilipes* (Costa Rica, Panama), *Hypolobocera bouvieri angulata*, *Orthothelphusa holthuisi* (Colombia, Venezuela), and *Hypolobocera exuca*, *Lindacatalina latipenis*, *L. orientalis*, and *L. sumacensis* (all Colombia, Ecuador), *Fredius estevisi* and *F. platyacanthus* (Brazil, Venezuela), *Kingsleya siolii* (Brazil, Suriname), *Zilchia aspoekorum* (Belize, Mexico), and *Raddaus tuberculatus* (Guatemala, Mexico).

The majority of pseudothelphusids (22 species) that have a widespread distribution exceeding 20,000 km^2 are assessed as Least Concern. However, there are some species with a small distributional range that spans the border between two neighboring countries that are assessed as either Vulnerable: *Hypolobocera exuca* (Colombia, Ecuador), *Kingsleya siolii* (Brazil, Suriname) and *Zilchia aspoekorum* (Belize, Mexico), or Data Deficient: *Fredius platyacanthus* (Brazil, Venezuela), *Lindacatalina latipenis* and *L. sumacensis* (both Colombia, Ecuador), *Orthothelphusa holthuisi* (Colombia, Venezuela), and *Ptychophallus exilipes* (Costa Rica, Panama).

Widespread Species: Trichodactylidae

Some 8 out of 15 trichodactylid freshwater crab genera and 20 out of 47 species have a wide distributional range exceeding 20,000 km^2 that spans more than one country: all are assessed as Least Concern. Notably, *Valdivia serrata* is found in nine countries (Bolivia, Brazil, Colombia, Ecuador, Peru, Venezuela, Guyana, Suriname, French Guiana), *Poppiana dentata* in eight countries (Brazil, Colombia, French Guiana, Guyana, Nicaragua, Suriname, Trinidad and Tobago, Venezuela), and *Sylviocarcinus pictus* in seven countries (Colombia, Brazil, Bolivia, Peru, Argentina, French Guiana, Guyana). *Dilocarcinus pagei* is found in six countries (Argentina, Brazil, Bolivia, Colombia, Paraguay, Peru), and *Sylviocarcinus devillei* and *Moreirocarcinus emarginatus* are both found in 5 countries (Bolivia, Brazil, Colombia, Ecuador, and Peru; and Brazil, Colombia, Ecuador, Peru, and Venezuela respectively). Four species are found in four countries: *Trichodactylus kensleyi* (in Argentina, Brazil, Paraguay and Uruguay) and *Poppiana argentiniana*, *Valdivia camerani*, and *Trichodactylus borellianus* (in Argentina, Bolivia, Brazil, and Paraguay). Seven species are found in three countries (*Rotundovaldivia latidens*, *Sylviocarcinus maldonadoensis*, *S. australis*, *Trichodactylus faxoni*, *T. panoplus*, *Valdivia cururuensis* and *Zilchiopsis oronensis*), four species are found in two countries (*Bottiella niceforei*, *B. cucutensis*, *Forsteria venezuelensis*, *T. quinquedentatus*), and three species are found in one country (*Trichodactylus petropolitanus*, *Valdivia novemdentata*, and *Zilchiopsis collastinensis*).

Distribution patterns and habitat

Freshwater crabs are found in all major habitat types in the Neotropics (Thieme et al. 2005; Abell et al. 2008) including rivers, rapids, swamps, lakes, caves, and mountain streams and species belonging to both families prefer warm-water habitats year round. The Pseudothelphusidae are typically found in small mountain streams above 400 m above sea level, which may in part reflect their ability to breathe air and live a semiterrestrial existence. Pseudothelphusids reach their highest diversity in the rivers and streams that drain the highland areas of the cordilleras of Colombia and Ecuador. In contrast, trichodactylids are most abundant in the vast network of lowland rivers of South America that make up the Amazon, Orinoco, Paraguay, and Parana basins (Cumberlidge et al. 2009). Species diversity tends to be highest where vegetation cover is densest and water availability is highest, and there are fewer, or no species found in the more arid ecosystems and those with colder water temperatures. The pattern of the dominance of Pseudothelphusidae in Colombia, Venezuela, Ecuador, Peru, Mexico, Central America, and the Caribbean, and their absence in Bolivia, Uruguay, Paraguay, southern Argentina, and Chile probably reflects an origin somewhere in Colombia and Venezuela, from where they spread out north to Central America and Mexico, east to the Guyanas and Lesser Antilles, and south as far as Brazil and Peru (Rodríguez 1982, 1986).

Similarly the dominance of Trichodactylidae in the Amazon, Orinoco, Paraguay, and Parana River basins and their absence from Chile and from aquatic ecosystems at high altitudes in the Andes elsewhere in South America reflects their preference for an aquatic, rather than a semiterrestrial life (Rodríguez 1982, 1986). However, it is harder to explain the presence of trichodactylids in Central America, Mexico, and Trinidad in localities that lie well outside of the major river basins of the South American subcontinent (Rodríguez 1992).

Twenty-four species of neotropical freshwater crabs are found in caves (22 pseudothelphusids and two trichodactylids), and are either fully adapted to a life in caves, or are visitors to caves and have a distribution that also includes surface waters (Table 3). Twelve species of cave-dwelling freshwater crabs are from Mexico, five from Central America (Belize 1, Guatemala 3, and Costa Rica 1), two from the Caribbean (Cuba 1, Puerto Rico, and St. Croix 1), and five from South America (Colombia, 2 and Venezuela 3).

Table 3. Species of Neotropical freshwater crabs that are associated with caves, and the country where each species occurs. The family, subfamily/tribe for each species are also shown, together with its IUCN Red List conservation status. LC = Least Concern, EN = Endangered, DD = Data Deficient. For explanation of Red List categories see IUCN (2003). * = recently described species, not assessed yet for conservation status.

Family	Tribe	Species	Country	Cons. Status
Pseudothelphusidae	Strengerianini	*Neostrengeria charalensis*	Colombia	LC
Pseudothelphusidae	Strengerianini	*Neostrengeria sketi*	Colombia	DD
Pseudothelphusidae	Hypolobocerini	*Achlidon puntarenas*	Costa Rica	DD
Pseudothelphusidae	Epilobocerinae	*Epilobocera cubensis*	Cuba	LC
Pseudothelphusidae	Hypolobocerini	*Phrygiopilus acanthophallus*	Guatemala	DD
Pseudothelphusidae	Potamocarcinini	*Typhlopseudothelphusa juberthiei*	Guatemala	DD
Pseudothelphusidae	Potamocarcinini	*Zilchia falcata*	Guatemala	DD
Pseudothelphusidae	Potamocarcinini	*Typhlopseudothelphusa acanthochela*	Belize	DD
Trichodactylidae	Trichodactylinae	*Avotrichodactylus constrictus*	Mexico	LC
Trichodactylidae	Trichodactylinae	*Rodriguezia mensabak*	Mexico	DD
Pseudothelphusidae	Hypolobocerini	*Phrygiopilus montebelloensis*	Mexico	DD
Pseudothelphusidae	Hypolobocerini	*Phrygiopilus yoshibensis*	Mexico	DD
Pseudothelphusidae	Potamocarcinini	*Odontothelphusa monodontis*	Mexico	DD
Pseudothelphusidae	Potamocarcinini	*Potamocarcinus leptomelus*	Mexico	DD
Pseudothelphusidae	Pseudothelphusini	*Pseudothelphusa mexicana*	Mexico	DD
Pseudothelphusidae	Pseudothelphusini	*Tehuana complanata*	Mexico	DD
Pseudothelphusidae	Potamocarcinini	*Typhlopseudothelphusa hyba*	Mexico	DD
Pseudothelphusidae	Potamocarcinini	*Typhlopseudothelphusa mocinoi*	Mexico	EN
Pseudothelphusidae	Potamocarcinini	*Sylvathelphusa cavernicola*	Mexico	*
Pseudothelphusidae	Potamocarcinini	*Villalobosus lopezformenti*	Mexico	DD
Pseudothelphusidae	Epilobocerinae	*Epilobocera sinuatifrons*	Puerto Rico	LC
Pseudothelphusidae	Strengerianini	*Chaceus caecus*	Venezuela	DD
Pseudothelphusidae	Strengerianini	*Chaceus turikensis*	Venezuela	DD
Pseudothelphusidae	Pseudothelphusini	*Rodriguezus garmani*	Venezuela	LC

Freshwater crab endemism (Table 4)

The majority of freshwater crab species found in Colombia (81%) are country endemics: 78 species of pseudothelphsids and 5 species of trichodactylids (Cumberlidge et al. 2009). The high degree of endemism in Colombia's freshwater crab fauna at the species level (83 of 102 species, 81%) is also seen to a lesser extent at the genus level (9 of 21 genera, 43%), but not at the family level (neither of the two families are exclusively found in Colombia). Many of the Colombian endemics are found in the isolated mountain streams and in the middle stretches of rivers associated with rainforest (Campos 2005). Mexico's freshwater crab fauna has the next highest number of country endemics (66 of 67 species, 97%) that is also seen at the genus level (13 of 17 genera, 76%), but not at the family level (neither of the two families are exclusively found in Mexico). Many of the Mexican endemics are found in the isolated mountain streams and in the middle stretches of rivers associated with the rainforests and caves

Table 4. Countries that host endemic species of Neotropical freshwater crabs. First number indicates the number of species of Pseudothelphusidae (P), the second number indicates the same data for the Trichodactylidae (T).

Country	No. Endemic Sp. (P, T)
Colombia	78, 5
Mexico	62, 4
Venezuela	22, 0
Panama	12, 1
Ecuador	11, 0
Costa Rica	9, 0
Cuba	9, 0
Guatemala	9, 0
Brazil	7, 10
Peru	6, 3
El Salvador	3, 0
Nicaragua	3, 0
Belize	2, 0
Dominican Republic	2, 0
Guyana	2, 0
Haiti	1, 0
Honduras	1, 0
Suriname	1, 0
Trinidad	1, 0
Bolivia	0, 1

(Villalobos and Alvarez 2010). All species of freshwater crabs found in Cuba and Hispaniola are island endemics.

Conservation status

The conservation status of the 298 species of freshwater crabs known from the Neotropical region in 2009 was evaluated against the IUCN (2003) Red List criteria (version 3.1) and is available on the Red List site (www.iucn.redlist.org). These results were summarized by Cumberlidge et al. (2009). The conservation status of species described since that work (Table 1) has not yet been assessed. Unfortunately, about half of all Neotropical freshwater crabs (148 species) are too poorly known to submit to the assessment protocols and are listed as Data Deficient, notably some 80% of the diverse Mexican fauna (Tables 5, 6). Of the remaining 150 species that could be assessed (belonging to 22 genera and 2 families) the majority (107 species) were found to be of Least Concern, and most of these species either live in rivers, marshy lowlands, or in mountain streams in the forested highlands (Cumberlidge et al. 2009). For the Pseudothelphusidae, 111 out of 251 species were assessed (140 species were data deficient), and of these, 72 species of Least Concern, 2 species were Critically Endan-

Table 5. Summary of the Red List assessments of the two families of Neotropical freshwater crabs based on data from Cumberlidge et al. (2009). Assessed = number of species that could be assessed using the IUCN Red List protocols; Thr. = total number of species assessed in one of the threatened categories (VU = Vulnerable, EN = Endangered, CR = Critically Endangered); LC = Least Concern, NT = Near Threatened, DD = Data Deficient. For explanation of Red List categories see IUCN (2003).

Family	Total Sp.	Assessed	Thr.	Thr. (%)	VU	EN	CR	LC	NT	DD
Pseudothelphusidae	251	111	38	34.2	33	3	2	72	1	140
Trichodactylidae	47	39	4	10.3	3	1	0	35	0	8
Total	298	150	42	28.3	36	4	2	107	1	148

Table 6. Summary of the countries in the Neotropical region that have threatened species of freshwater crabs based on data from Cumberlidge et al. (2009). Assessed = number of species that could be assessed using the IUCN Red List protocols; Thr. = total number of species assessed in one of the threatened categories (VU = Vulnerable, EN = Endangered, CR = Critically Endangered); LC = Least Concern, NT = Near Threatened, DD = Data Deficient.

Country	Total Sp.	Assessed	Thr.	Thr. (%)	VU	EN	CR	LC	NT	DD
Colombia	101	60	14	23.3	13	0	1	45	1	41
Venezuela	42	31	7	22.6	7	0	0	24	0	11
Mexico	63	19	6	31.6	2	3	1	13	0	44
Ecuador	27	19	5	36.8	5	0	0	12	0	8
Peru	25	21	3	14.3	3	0	0	0	0	4
El Salvador	8	6	2	33.3	2	0	0	4	0	2
Costa Rica	13	7	2	28.6	2	0	0	5	0	6
Brazil	45	38	2	10.5	1	1	0	34	0	7
Suriname	11	10	1	10.0	1	0	0	0	0	1
Honduras	6	6	1	33.3	1	0	0	4	0	0
Haiti	1	1	1	100.0	1	0	0	0	0	0
Guatemala	13	7	1	28.6	1	0	0	5	0	6
Dominican Rep.	2	2	1	100.0	1	0	0	0	0	0
Cuba	9	9	1	11.0	1	0	0	0	0	0

gered, 3 species were Endangered Critically Endangered, 3 species were Endangered, and 33 species were Vulnerable (Tables 5, 6). For the Trichodactylidae, 39 out of 47 species were assessed (8 species were Data Deficient), and of these, 35 species were of Least Concern, one species was Endangered, and three were Vulnerable (Table 6). For both families combined, 42 of the 150 assessed species of Neotropical freshwater crabs (28%) were listed in one of three threatened categories, either as Critically Endangered (2 species), Endangered (4 species) or Vulnerable (36 species) (Table 7). One species was assessed as near threatened, and no species of Neotropical freshwater crabs was confirmed as Extinct or Extinct in the Wild. However, it should be noted that a species cannot be formally assessed as extinct until exhaustive surveys probing its disappearance have been carried out. Because conservation status could not be assigned to large numbers of Data Deficient species (148), the proportion of threatened species in Cumberlidge

Table 7. Threatened species of Neotropical freshwater crabs. P = Pseudothelphusidae, T = Trichodactylidae. For explanation of Red list categories see IUCN (2003).

Family/Subfamily/Tribe	Taxon	IUCN Red List Category	Distribution
T. (Trichodactylinae)	*Trichodactylus crassus*	EN B1ab(iii)	Brazil
P. (Kingsleyini)	*Kingsleya siolii*	VU B1ab(iii); D2	Brazil, Suriname
P. (Kingsleyini)	*Fredius platyacanthus*	VU B1ab(iii); D2	Brazil, Venezuela
P. (Kingsleyini)	*Fredius stenolobus*	VU B1ab(iii); D2	Brazil, Venezuela
P. (Hypolobocerini)	*Hypolobocera alata*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Hypolobocera andagoensis*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Hypolobocera barbacensis*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Hypolobocera cajambrensis*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Hypolobocera rotundilobata*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Hypolobocera velezi*	VU B1ab(iii); D2	Colombia
P. (Hypolobocerini)	*Moritschus altaquerensis*	VU B1ab(iii); D2	Colombia
P. (Kingsleyini)	*Fredius granulatus*	VU B1ab(iii); D2	Colombia
P. (Strengerianini)	*Chaceus ibiricensis*	VU B1ab(iii)+2ab(iii); D2	Colombia
P. (Strengerianini)	*Strengeriana antioquensis*	CR B1ab(i,ii,iii,iv,v)+2ab(i,ii,iii,iv,v)	Colombia
T. (Dilocarcininae)	*Bottiella medemi*	VU B1ab(iii)+2ab(iii)	Colombia
P. (Hypolobocerini)	*Hypolobocera exuca*	VU B1ab(iii); D2	Colombia, Ecuador
P. (Hypolobocerini)	*Lindacatalina sumacensis*	VU B1ab(iii); D2	Colombia, Ecuador
T. (Dilocarcininae)	*Bottiella cucutensis*	VU B1ab(iii)+2ab(iii	Colombia, Venezuela
P. (Potamocarcini)	*Allacanthos pittieri*	VU B1ab(iii); D2	Costa Rica
P. (Potamocarcini)	*Ptychophallus tristani*	VU B1ab(iii)+2ab(iii); D2	Costa Rica
P. (Epilobocerinae	*Epilobocera wetherbeei*	VU B1ab(iii); D2	Dominican Republic
P. (Hypolobocerini)	*Hypolobocera delsolari*	VU B1ab(iii); D2	Ecuador
P. (Hypolobocerini)	*Hypolobocera rathbuni*	VU B1ab(iii); D2	Ecuador
P. (Hypolobocerini)	*Moritschus ecuadorensis*	VU B1ab(iii); D2	Ecuador
P. (Hypolobocerini)	*Elsalvadoria zurstrasseni*	VU B1ab(iii)+2ab(iii); D2	El Salvador
P. (Hypolobocerini)	*Raddaus mertensi*	VU D2	El Salvador, Guatemala, Honduras
P. (Hypolobocerini)	*Phrygiopilus acanthophallus*	VU B1ab(iii); D2	Guatemala
P. (Epilobocerinae)	*Epilobocera haytensis*	VU B1ab(iii)	Haiti, Dominican Rep.
P. (Potamocarcini)	*Potamocarcinus roatensis*	VU D2	Honduras
P. (Potamocarcini)	*Potamocarcinus hartmanni*	VU B1ab(ii)+2ab(ii); D2	Mexico
P. (Potamocarcini)	*Typhlopseudothelphusa mocinoi*	EN B1ab(iii)+2ab(iii)	Mexico
P. (Pseudothelphusini)	*Tehuana lamothei*	EN B1ab(ii,iii)+2ab(ii,iii)	Mexico
P. (Pseudothelphusini)	*Tehuana poglayenorum*	EN B1ab(ii,iii)+2ab(ii,iii)	Mexico
P. (Pseudothelphusini)	*Tehuana veracruzana*	CR B1ab(i,ii,iii,iv,v)+2ab(i,ii,iii,iv,v)	Mexico
T. (Trichodactylinae)	*Avotrichodactylus oaxensis*	VU B1ab(iii)+2ab(iii)	Mexico
P. (Hypolobocerini)	*Hypolobocera gracilignatha*	VU D2	Peru
P. (Hypolobocerini)	*Hypolobocera lamercedes*	VU B1ab(iii); D2	Peru
P. (Hypolobocerini)	*Hypolobocera peruviana*	VU D2	Peru
P. (Kingsleyini)	*Microthelphusa forcarti*	VU B1ab(iii); D2	Venezuela
P. (Kingsleyini)	*Neopseudothelphusa fossor*	VU B1ab(iii); D2	Venezuela
P. (Kingsleyini)	*Rodriguezus trujillensis*	VU B1ab(iii)+2ab(iii); D2	Venezuela

et al. (2009) (32%) is almost certainly an underestimation. This means that the number of threatened species could well increase should a Data Deficient species subsequently be assessed as threatened (Cumberlidge et al. 2009). Despite the large numbers of specimens available very little information is available on population levels and trends, except for a qualitative estimate (e.g., whether common or rare) based on the number of sites at which a species is present and its relative abundance at each site.

With only 42 out of 298 species of Neotropical freshwater crabs known in 2009 assessed as threatened with global extinction (Tables 5–7), the region's largely endemic freshwater crab fauna (Table 4) does not appear at first sight to be in immediate trouble compared with other aquatic groups found in the same freshwater habitats (e.g., fish, molluscs, and dragonflies) (Collen et al. 2008). The 107 species of Neotropical freshwater crabs judged to be of Least Concern (35 species of trichodactylids, and 72 species of pseudothelphusids) have a wide distribution in rivers, lakes, highland streams and lowland wetlands, and appear to be tolerant of changes in land use that affect aquatic ecosystems.

Threats to Neotropical freshwater crabs include habitat destruction driven by increasing agriculture, the demands of increasing industrial development, and the alteration of fast flowing rivers for the creation of hydroelectric power (Cumberlidge et al. 2009). Even species assessed as Least Concern could suffer a catastrophic decline should there be abrupt changes in land development, hydrology, or pesticide-use regimes. Species with a narrow distribution are vulnerable to extreme population fragmentation and could suffer a rapid decline and even extinction in a relatively short time should dramatic changes in land-use affect their habitat. The 148 species of Neotropical freshwater crabs judged to be Data Deficient are mostly rare species, and their conservation status needs to be re-evaluated once more information comes to light. It is hoped that prioritizing species for conservation action through the Red List assessment process will lead to the development of conservation recovery plans for threatened species in the future.

Conclusions

This study confirms that freshwater crabs are completely absent from all parts of the Nearctic region (even in the neighboring warmer parts of northern Mexico and southern Florida, USA), and that the northern limits of their distribution coincide with the Nearctic/Neotropical boundary in Mexico and the presence of arid regions lacking permanent freshwaters (i.e., the Sonoran and Chihuahuan Deserts). Species richness of freshwater crabs increases south of this boundary in Mexico and reaches a peak in the States of Veracruz, Tabasco, Oaxaca, and Chiapas. The uneven pattern of species-richness – high in Mexico, low in Central America, and high in Colombia – is difficult to explain by simply invoking an even spread of species out of a center of origin somewhere in Colombia –Venezuela (Rodríguez 1982, 1992), and further studies are necessary. The relatively low species richness of freshwater crabs in Brazil is unexpected given its vast area and rich biodiversity, and may reflect the dominance of trichodactylids and a general paucity of species-rich pseudothelphusids in the Brazilian fauna. The abundance of freshwater crabs in Colombia, Ecuador, and Peru coincides with a freshwater habitat species diversity hotspot (that is defined by overlapping distributions of stenotopic species based on combined data from vertebrates and invertebrates (Collen et al. 2008)). All countries in the Neotropical region are still in a phase of exploration and steep rises in species numbers of pseudothelphusids are expected once more surveys have been completed.

The Neotropics are the second most diverse region in the world for freshwater crabs, and there are 42 species from 14 countries within this region that may be threatened with extinction. The finding that 34% of pseudothelphusids and 10% of trichodactylids are threatened, and that many others are poorly known, can be used to develop a conservation strategy for these species, and supply a focus for future research efforts (Cumberlidge et al. 2009). Although some species of Neotropical freshwater crabs have been quite well studied a clear majority are known only from either the type locality or from just a few localities, and in these cases further collections are necessary to ascertain their actual distributions. The restricted range of many species from the Neotropics, together with the on-going human induced loss of habitat in many parts of the region, are a cause for concern for the long-term security of elements of this fauna. Conservation activities should therefore be aimed primarily at preserving the integrity of sites and habitats while at the same time closely monitoring key populations. Significant areas of the Neotropics still remain insufficiently explored, and the focus of new efforts should be on new species discovery through increased collection in remote areas and the refinement of taxonomic skills. These efforts should also focus on conserving threatened species, and on seeking out data deficient species. Gathering current data on distribution, natural history, population trends, threats, and endemism of the Neotropical region's highly diverse freshwater crabs will enable the updating of the IUCN Red Lists, and the development of conservation strategies for this understudied diverse and potentially highly threatened fauna.

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Relative growth and morphological sexual maturity size of the freshwater crab *Trichodactylus borellianus* (Crustacea, Decapoda, Trichodactylidae) in the Middle Paraná River, Argentina

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Abstract

The relative growth of a number of morphological dimensions of the South American freshwater crab *Trichodactylus borellianus* (Trichodactylidae) were compared and related to sexual dimorphism. Crabs were collected from ponds in the Middle Paraná River in Argentina. A regression model with segmented relationship was used to test for relative growth between these measurements where breakpoints infer the body size at which crabs reach sexual maturity. In both sexes the carapace width and the length, height, and thickness of the right and left chelae were measured, as well as the male pleopod length and the female abdomen width. All of these measurements were found to show positive allometry with the exception of the male pleopod length and the left chelae, which did not show a breakpoint. In females the breakpoint for the abdomen width inferred a morphological sexual maturity at carapace width 6.9 mm. In males the break point for the pleopod length was at carapace width 6.6 mm, with that for the chelae measurements was between carapace widths 6.4 and 6.9 mm. The relative growth pattern in *T. borellianus* was found to be similar to that recorded for other species of the family Trichodactylidae.

Keywords

Chelipeds, cephalothorax width, pleopods, reproduction, growth, regression model

Introduction

The onset of maturity in crustaceans is signaled by a series of morphological, physiological and behavioral transformations through which immature individuals become able to produce reproductive cells and copulate (Hartnoll 1969). When organisms grow some of their morphological dimensions increase at a different rate from others that results in a change in body proportions known as relative growth (Hartnoll 1978). Relative growth data have been widely used to predict the onset of morphometric sexual maturity in a number of organisms (Haley 1969). Here we report on the results of a similar study on a species of South American freshwater crab of the family Trichodactylidae.

The Trichodactylidae is a neotropical family of freshwater crabs found throughout the river basins of tropical and subtropical South America, with the exception of the Pacific slope rivers (Magalhães 2003). Morphological maturity in the Trichodactylidae can be estimated by taking measurements of the characters of secondary sexual characters such as the male pleopods (gonopods), male chelipeds, and the female abdomen. External morphological changes that signal sexual maturity in freshwater crabs can be used to determine whether crabs are adult or immature (Somerton 1980, Somerton and MacIntosh 1983, Conan and Comeau 1986). This knowledge has been used by researchers to both identify species and to describe population structure and dynamics (Haley 1969, Hartnoll 1978, Vannini and Gherardi 1988, Felder and Lovett 1989, Chow and Sandifer 1991, Pinheiro and Fransozo 1998, Robertson and Butler IV 2003, Lima et al. 2013).

There are only a few recent studies of the allometric changes associated with growth within the genus *Trichodactylus* (Venâncio and Leme 2010, Lima et al. 2013, Silva et al. 2014). The present work focuses on allometric growth in *Trichodactylus borellianus* Nobili, 1896, which is a common species associated with the aquatic vegetation of the Paraná alluvial system in Argentina where it occurs in the Paraná and Paraguay rivers (Collins et al. 2007), as well as in Brazil (Pará, Mato Grosso, Mato Grosso do Sul), Bolivia, and Paraguay (Magalhães 2003). Previous studies on *T. borellianus* have analyzed the influence of temperature on growth (Renzulli and Collins 2000), but the allometric changes associated with growth and the size at which it reaches sexual maturity are still unknown. A knowledge of the allometric growth of a species can be an important tool for assessing geographical variation in widespread species as *T. borellianus* (see Hines 1989). The aim of the present study was to describe allometric growth and to estimate the onset of morphological sexual maturity of *T. borellianus* using a series of body dimensions, including those associated with secondary sexual characters.

Methods

Monthly collections were carried out during the day for fourteen months from August 2001 to October 2002. The samples were taken from three sites of the Paraná alluvial valley: Las Sandias Stream (S31°41'15.3"W 60°31'31.6"), Aliviador Stream (S31°40'17.9", W60°34'45.9") and Santa Fe River (S 31°38'35", W60°40'05.5"). The latter river is the biggest channel of La Plata Basin, containing 85% of the total freshwater in Argentina. The Paraná River system consists of a main channel with an alluvial valley ranging from approximately 13 to 56 km in width with a slope of 0.036 m km^{-1}. The main channel is located on the eastern margin and is between 2 and 4.2 km wide, and is 2.3 km wide at the study site. The western area of the river has several secondary rivers, streams, ponds and islands, extending approximately 10 km in width to the main channel. The flow along the main channel varies between 10,600 m^{-3}s^{-1} and 31,000 m^{-3}s^{-1} causing a primary runoff in spring and summer (November to March), which originates annual floods. Autumn and winter are the low water seasons (Bonetto and Wais 1995).

In the laboratory, a total of 337 crabs were analyzed of which 155 were males and 182 females. The following variables were recorded: carapace width (CW), right cheliped length (RChL), right cheliped height (RChH) and right cheliped width (RChW), left cheliped length (LChL), left cheliped height (LChH) and left cheliped width (LChW), abdomen width (AW) in females, and left first pleopod length (PL) in males (Fig. 1; Table 1). Organisms were measured with digital calipers to the nearest 0.01 mm, and only intermoult-stage organisms were used. Specimens with injured chela, with regenerating or missing chelipeds, or injury and/or malformation in other structures were excluded.

The CW was used as the independent variable to test relative growth in the other measurements (the dependent variables). Linear regressions, comparisons of slopes, and calculations of the onset of morphological sexual maturity were made with the software R version 2.13.2 (R development core team 2011). To analyze the onset of morphological sexual maturity a Regression Model with Segmented Relationship with breakpoint with the package 'segmented' was used in the regressions that visualized possible breakpoints (Muggeo 2003, 2008). This model is based on regression models where the relationships between the response and one or more explanatory variables are piecewise linear, namely represented by two or more straight lines connected at breakpoints (Muggeo 2003). Package segmented estimates linear and generalized linear models having one or more segmented relationships in the linear predictor. Estimates of the slopes and of the possibly multiple breakpoints are provided. The package includes testing/estimating functions and methods to print, summarize, and plot the results (Muggeo 2008).

The Davies' test was used to test for significant differences of slopes between juveniles and adults and to test for a non-constant regression parameter in the linear predictor (Davies 1987). The slopes of the first segment (juveniles) and second segment (adults), when the Davies' test was significant were estimated with the results summary of the Regression Model with Segmented Relationship with breakpoint (Muggeo 2003). A slope value = 1 represents isometric growth, a slope value < 1 represents negative allometric growth, and a slope value > 1 represents positive allometric growth.

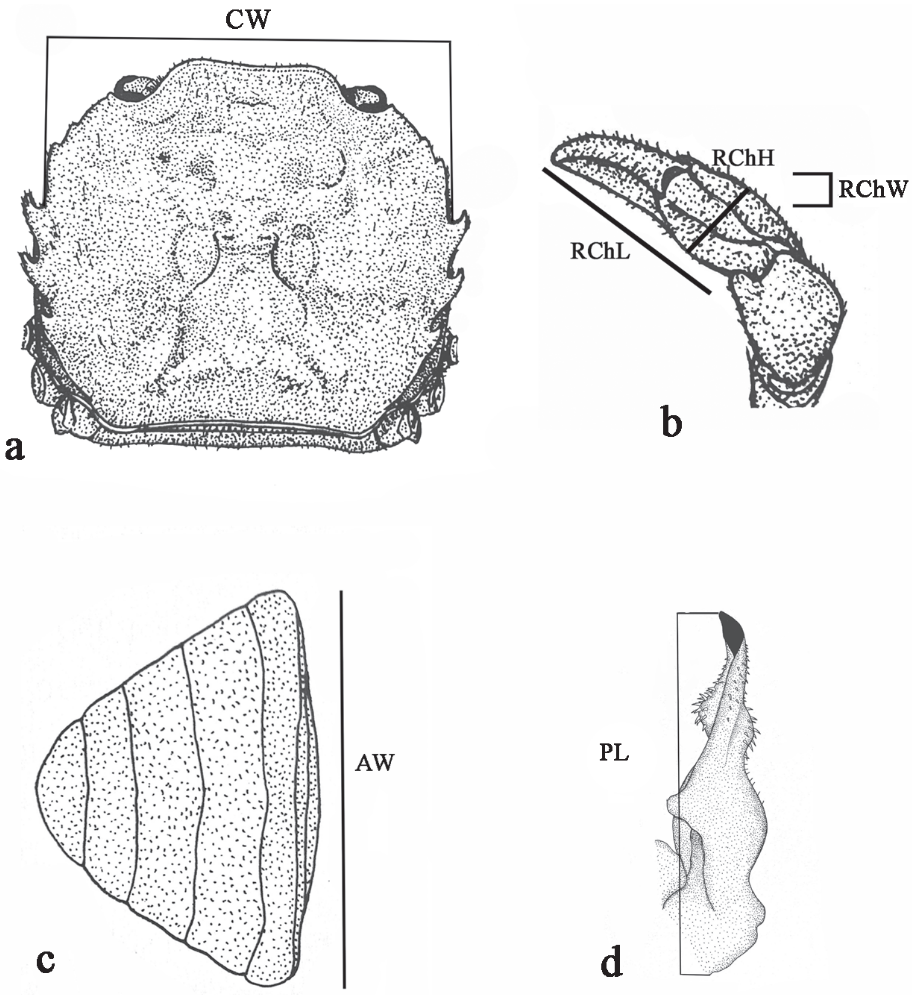

Figure 1. Measures taken of the crab *Trichodactylus borellianus* (modified from Magalhães 2003): **a** carapace width (CW) **b** right cheliped length (RChL), right cheliped height (RChH) and right cheliped width (RChW) **c** third abdominal width of females (AW) **d** left pleopod length (PL).

Table 1. Characterization of how the morphometric variables were obtained. CW: carapace width, ChL: cheliped length, ChH: cheliped height, ChW: cheliped width, AW: abdomen width, PL: pleopod length.

Body part (morphometric variable)	Measuring mode
CW	Distance between the first postorbital spines of the carapace
ChL	Ventral distance between distal end of the propodus and the carpus articulation
ChH	Maximum propodus thickness
ChW	Maximum distance between lateral margins of the propodus
AW	Maximum distance of the third segment of female abdomen
PL	Maximum length of the left pleopod

Results

Male crabs had a CW of 6.34 ± 1.48 mm (ranging from 2.9 to 10.4 mm CW); female crabs had a CW of 6.12 ± 1.92 mm (ranging from 2.7 to 12.4 mm). There were no statistically significant differences between the CW of male and female crabs (t= -1.21; *p* = 0.227). The regression using CW as the independent variable indicated that measures of the right cheliped of male crabs were best adjusted to two straight lines rather than to one because these presented statistically significant differences between the slopes of both lines (Fig. 2, Table 2). However, a breakpoint was found on PL (Fig. 2) despite it not being well adjusted to the regression model and the Davies' test finding no statistical significance (Table 2). The breakpoints produced by segmented regression ranged from CWs 6.5 to 6.9 mm for the measurements of the right chelipeds. The onset of morphological maturity size (breakpoint) for PL was similar to RChL (~CW 6.5 mm) (Table 2) (Fig. 2). No observed possible breakpoints were found for the regressions of the left cheliped in male crabs (Table 2). Juvenile and adult stages of male

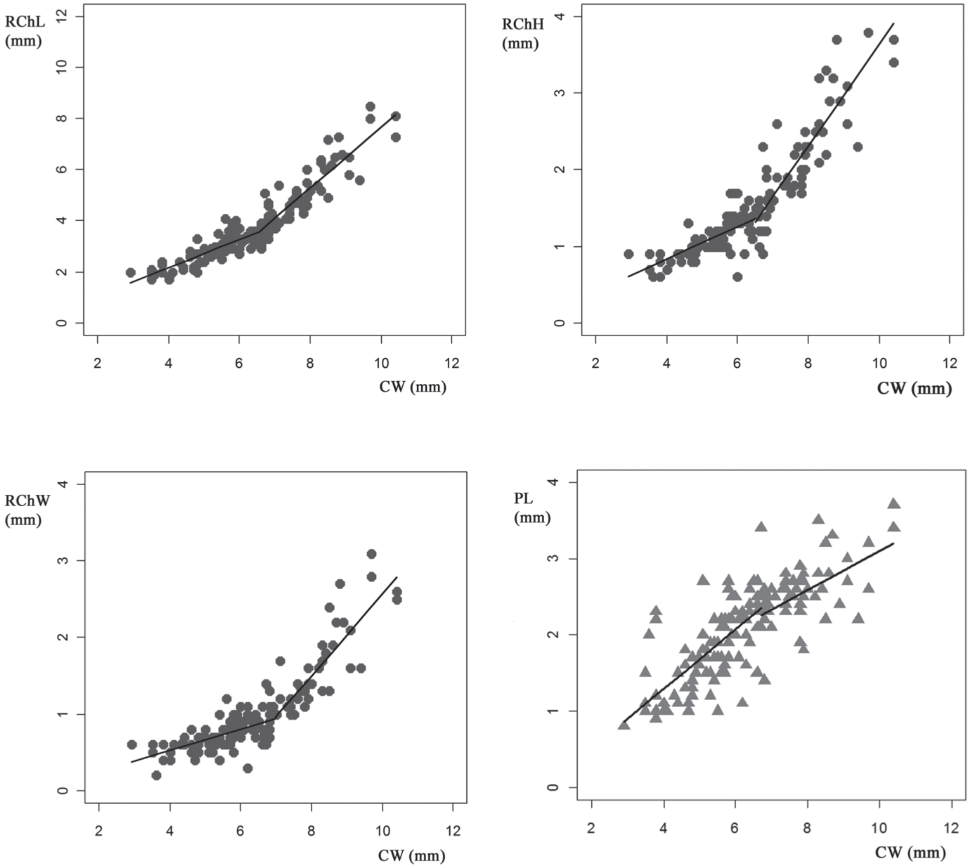

Figure 2. Segmented Relationship with breakpoint on male crabs of *Trichodactylus borellianus*. RChL: right cheliped length; RChH: right cheliped height; RChW: right cheliped width; PL: left pleopod length.

Table 2. Results of Regression Model with Segmented Relationship with breakpoint studied for males and females of *T. borellianus* with Davies' test for change in the slope, This includes the estimate slopes for J: juveniles and A: adults.

Sexes	Relationship	Estimated break point CW (mm)	R-squared	Intercept	Davies' test for change in the slope p-value	Stage	Slopes	Allometry
Males	RChL vs. CW	6.6	0.89	-0.02	<0.0001	J	0.55	-
						A	1.19	+
	RChH vs. CW	6.5	0.85	-0.01	<0.0001	J	0.21	-
						A	0.66	-
	RChW vs.CW	6.9	0.82	-0.02	<0.0001	J	0.14	-
						A	0.54	-
	PL vs. CW	6.6	0.60	-0.24	0.2836	J	0.39	-
						A	0.25	-
	LChL vs. CW	(*)	0.81	0.89	without slope change	-	-	
	LChH vs. CW	(*)	0.73	0.25	without slope change			
	LChW vs. CW	(*)	0.53	0.19	without slope change			
Females	RChL vs. CW	6.0	0.90	0.66	<0.0001	J	0.37	-
						A	0.62	-
	RChH vs. CW	(*)	0.85		without slope change			
	RChW vs.CW	(*)	0.70		without slope change			
	AW vs. CW	6.9	0.91	-0.79	<0.0001	J	0.73	-
						A	1.30	+
	LChL vs. CW	5.7	0.91	0.54	<0.0001	J	0.37	-
						A	0.68	-
	LChH vs. CW	(*)	0.82		without slope change			
	LChW vs. CW	(*)	0.66		without slope change			

crabs showed negative allometric relationships between RChH, RChW, PL and CW. The slopes of the adult measurements of the right cheliped were higher than those of juveniles, and were lower in adults than juveniles for PL (Table 2, Fig. 2). The RChL vs. CW on adult male crabs showed positive allometric relationships in contrast with juveniles that presented negative allometric relationships (Table 3). Adult crabs were better adjusted to the regressions than juvenile crabs (Table 2).

In females, there were three characters that best adjusted to two straight lines rather than to one: the lengths of both chelae and the abdomen width (Table 2, Fig. 3). These presented statistically significant differences between the slopes of both lines (Table 2). Possible breakpoints were not visualized on the other measures of right and left chelipeds of female crabs (Table 2). Segmented regressions produced a breakpoint at 6.02 mm CW for RChL and a lower breakpoint for LChL (5.7 mm CW). The breakpoint of AW was somewhat higher than the cheliped lengths (Table 2). The regressions of juveniles and adult female crabs showed negative allometric relationships for RChL and LChL, and the slopes of adult measurements were higher than those of juveniles (Fig. 3, Table 3). The AW of adults showed a positive allometric relationship with CW in contrast to juveniles that presented negative allometric relationships (Fig. 3, Table 3).

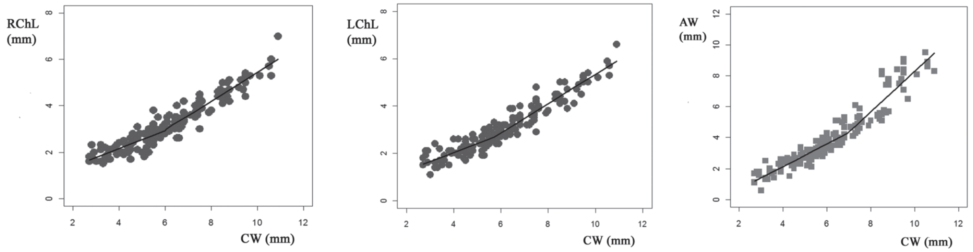

Figure 3. Segmented Relationship with breakpoint on female crabs of *Trichodactylus borellianus*. RChL: right cheliped length; LChL: left cheliped length; AW: third abdominal width.

Table 3. Results of allometric relationship study of each stage of *Trichodactylus borellianus*. J: juveniles; A: adults.

Sexes	Relationship	Stage	Linear equation *y= a + bx*	R-squared	Allometry
Males	RChL vs. CW	J	RChL = -0.0179 + 0.5478 CW	0.66	–
		A	RChL = -4.2291 + 1.1899 CW	0.80	+
	RChH vs. CW	J	RChH= -0.0109 + 0.2117 CW	0.50	–
		A	RChH = -3.003 + 0.6643 CW	0.76	–
	RChW vs. CW	J	RChW = -0.0217 + 0.1378 CW	0.40	–
		A	RChW = -2.7944 + 0.537 CW	0.68	–
	PL vs. CW	J	PL = -0.3109 + 0.3938 CW	0.46	–
		A	PL = 0.8324 + 0.2228 CW	0.28	–
Females	RChL vs. CW	J	RChL = 0.6661 + 0.3747 CW	0.59	–
		A	RChL = -0.7479 + 0.6187 CW	0.84	–
	AW vs. CW	J	AW = -0.7905 + 0.7309 CW	0.79	–
		A	AW = -4.7357 + 1.3027 CW	0.73	+
	LChL vs. CW	J	LChL = 0.5427 + 0.3747 CW	0.56	–
		A	LChL = -0.9007 + 0.6268 CW	0.87	–

Discussion

The knowledge of the life history of a species involves understanding such aspects as the development of sexual maturity, changes in allometric growth, and the age at which each of these occur. In crabs, morphological maturity is often observed together with allometric changes in growth (González-Gurriarán and Freire 1994, Fernández-Vergaz et al. 2000).

The results obtained in this study are in line with the points proposed by Hartnoll (1978) who showed that many crustaceans, particular brachyuran crabs, have differences in relative growth. For *T. borellianus* we also found this pattern, being able to identify male and female body parts that show morphological sexual maturity. Hartnoll (1974) believed that the growth of secondary sexual characters in crustaceans could be modeled by the power function. It was shown in this contribution that the relative growth rate differs between adults and juveniles, and that size at morphometric maturity can be estimated as the body size where the value changes. Also, in accordance with Hartnoll (1969), the population analyzed here shows that males and females maintain the same size, which favors the formation of mating pairs. The similarities in size between the sexes were also recorded for two other species of the same family (Mansur et al. 2005, Pinheiro and Taddei 2005). However, in geographically distant populations of *T. fluviatilis* the size of males and females showed differences, while in other populations there was no size difference (Lima et al. 2013, Silva et al. 2014).

For males, the three measurements around the right cheliped showed a good fit to the regression model, as well as differences in slopes of the regressions. These three measurements revealed a similar size from which growth patterns morphometrically change. According to Bueno and Shimizu (2009), the mean values of these three estimated measures can be adopted to represent the size at the onset of morphometric maturity, being in this case 6.6 mm CW. Otherwise, Davies' values for the PL were not significant and the R-squared values were not very high so this measure does not indicate a change in relative growth (Fig. 2d). Perhaps the pleopod width could be a better measure to indicate allometric growth than PL in this crab species. Cheliped measurements have been evaluated in many species as an indicator of relative growth and morphological sexual maturity. In males the cheliped, as an indicator of sexual maturity, could be a function of reproductive behavior (Mariappan et al. 2000, Shuster 2007). Crabs must become both functionally and physiologically mature before they can reproduce, and the ability to mate may depend on the attainment of morphometric maturity (Conan and Comeau 1986). While the reproductive behavior of this species has not yet been described, it can be suggested that *T. borellianus* could respond to patterns of other freshwater species. Thus, according to the proposal of Gherardi and Micheli (1989), this species could be mate in intermoult, and robust chelipeds in males would be needed to support females. On the other hand, it would be interesting to consider in future investigations the possibility of the existence of agonistic behavior in this freshwater crab.

Similar relative growth patterns were registered in other trichodactylids such as *Dilocarcinus pagei*, *Sylviocarcinus australis* and *Trichodactylus fluviatilis* (Mansur et al. 2005, Pinheiro and Taddei 2005, Lima et al. 2013, Silva et al. 2014). For *T. borellianus*, although the size range showing regression models are similar for both sexes, females show some traits that estimate maturity in different sizes. In this case, if the selected body part is LChL, females change growth patterns earlier than males. This pattern was also recorded in other brachyuran species (Negreiros-Fransozo et al. 2003, Lima et al. 2013). The size obtained with the regression model using AW vs. CW relationship is closer to the minimum size of ovigerous female (7.3 mm CW) recorded for this species (Senkman et al. in press). However, these records of ovigerous females may be underestimated due to the habits of these crabs that remain a long time in shelters and in roots of floating macrophytes (Magalhães 1986, Collins et al. 2006). Measuring the abdomen width as an indicator of relative growth and feature of morphological sexual maturity would have a relation with the incubation of eggs and the maternal care. The function of the abdomen in females as an incubator chamber is relevant in inland water species, related to the ability to incubate the eggs for a long time and to shelter the newly hatched juveniles until independence (Viau et al. 2006). For *T. borellianus* the capacity of the abdomen as an incubator chamber increased with increasing carapace width was demonstrated by Senkman et al. (in press).

Against this background, the changes in growth shown for the equations provide evidence of the reproductive function of the abdomen width in females and the chela in males. As in the crab *T. fluviatilis*, the relationship between RChL vs. CW also showed a positive allometry in both sexes (Lima et al. 2013, Silva et al. 2014). Allometry simply implies that the relative growth between the morphometric characters being studied is not constant. Relative to body size, chelae, abdomens, and first pleopods can all grow allometrically. Juvenile-to-adult changes in the relative growth rates of these characters can be used to identify the size of morphometric maturity (Teissier 1960). From a descriptive point of view, the AW and PL indicate that each sex may reach sexual maturity at different sizes; this pattern could be a possibility for the males to fertilise older females early.

For both sexes, the differences in the size of the chelae could involve some of different issues concerning reproduction. One of them could be related to the modification of feeding options. Variations in size of the chelae may also involve the possibilities of the range expansion of trophic items. This crab, found in the area of middle Paraná River, changes natural diet with body size (Williner and Collins 2013). This suggests a change in habitat. Larger claws could aid the crabs with changes in diet and predation that might accompany this habitat change. Adult crabs consume more plant debris and invertebrates of larger size than young crabs (Williner and Collins 2013). In turn, this diet change may correspond to a need in reproductive cell production (Nagaraju 2011). These possible associations between changes in the sizes of the chelipeds and changes in habitat and trophic potential have been recorded for the stone crabs (genus *Menippe*) from west-central Florida (Gerhart and Bert 2008).

We consider that the application of the Regression Model with Segmented Relationship with breakpoint with the package ‘segmented’ is an appropriate way to determine patterns of relative growth and sexual maturity. The present study also establishes the size range corresponding to the pubertal molt in this species. In this study, one might assume that, with the exception of traits that showed differential growth rates between adults and juveniles, for the remainder of the measured characters, that a shift in allometry between juveniles and subadults occurs in a continuous, gradual manner.

Considering the present contribution to the knowledge of reproductive traits, it is now necessary to assess sexual maturity in its other dimensions, mainly from the histological perspective, with a description of ducts and sexual cells. These results show that it is necessary to inquire about the sexual and mating system of this species in order to evaluate differences in measurements and trends found in this study (Baeza and Azorey 2012). On the other hand, as behavioral observations in the natural environment are difficult, it is recommended to raise crabs in the laboratory to evaluate mate selection and mating possibilities of different sizes.

The current study also provides a starting point for addressing additional aspects relevant to allometry of *T. borellianus*; however variations in allometry in other parts of the range of this species and among other populations still need to be evaluated.

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Morphological variation of freshwater crabs *Zilchiopsis collastinensis* and *Trichodactylus borellianus* (Decapoda, Trichodactylidae) among localities from the middle Paraná River basin during different hydrological periods

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Abstract

Measures of hydrologic connectivity have been used extensively to describe spatial connections in riverine landscapes. Hydrologic fluctuations constitute an important macrofactor that regulates other environmental variables and can explain the distribution and abundance of organisms. We analysed morphological variations among individuals of two freshwater crab species, *Zilchiopsis collastinensis* and *Trichodactylus borellianus*, from localities of the middle Paraná River basin during two phases of the local hydrological regime. Specimens were sampled at sites (localities) of Paraná River, Saladillo Stream, Salado River and Coronda River when water levels were falling and rising. The conductivity, pH, temperature and geographical coordinates were recorded at each site. The dorsal cephalothorax of each crab was represented using 16 landmarks for *Z. collastinensis* and 14 landmarks for *T. borellianus*. The Canonical Variate Analyses showed differences in shape (for both species) among the crabs collected from the Paraná and Salado Rivers during the two hydrologic phases. We did not find a general distribution pattern for shape among the crab localities. During falling water, the shapes of *Z. collastinensis* were not related to latitude-longitude gradient (i.e., showing greater overlap in shape), while during rising water the shapes were ordered along a distributional gradient according to geographical location. Contrary, shapes of *T. borellianus* were related to latitude-longitude during falling water and were not related to distributional gradient during rising water. The cephalothorax shape showed, in general, no statistically significant covariations with environmental variables for either species. These results show that each freshwater crab species, from different localities of the middle Paraná River, remain connected; however, these connections change throughout the hydrologic regime of the floodplain system. This study was useful for delineating how the relation among shapes of crabs of localities varies during two phases of the hydrological regime and for estimating the connections and geographical patterns in the floodplain system.

Keywords

Geometric morphometrics, Brachyura, floodplain, connectivity

Introduction

Measures of hydrologic connectivity have been used extensively to describe spatial connections in riverine landscapes (Ward 1989, Amoros and Bornette 2002). The dynamic and hierarchical nature of lotic ecosystems can be conceptualised as a four-dimensional fluvial hydrosystem, displaying distinct longitudinal, lateral, vertical and temporal characteristics (Ward 1989).

Floodplain systems vary broadly in their environmental characteristics and hydrological regimes (Ward et al. 2002). The alluvial valley, principally located in the middle and lower section of Paraná River, is a complex ensemble of lotic and lentic environments (Drago 2007). The main factor modelling the dynamics of the Paraná River system and its floodplain is the hydro-sedimentological cycle (Junk et al. 1989, Neiff 1990). Rising and falling water make two complementary phases of the cycle, which have much influence on the stability of river ecosystems (Neiff 1990). The period of flood is typically during spring and summer, while the period of low water is in autumn and winter (Drago 2007). In this sense, this hydrological fluctuation constitute an important macrofactor that regulates environmental variables and can explain the richness, distribution and abundance of organisms that live in these systems (Junk et al. 1989, Neiff et al. 2001, Mayora et al. 2013).

The movements of some species are related to the spatial and temporal dynamics of the floodplain system that they inhabit. Dispersal is defined as the movement of an organism over a specified distance or from one predefined patch to another (Bennetts et al. 2003). Depending on the hydrologic regime of the floodplain system, dispersal tends to homogenise populations between water bodies during high water periods and to decrease the differences between richness and abundance of organisms at nearby sites (Gomes et al. 2012). Freshwater invertebrates disperse through active or passive movements, which can influence colonisation rates, gene flow, and evolutionary divergence (Bilton et al. 2001).

The movements of freshwater decapods are influenced by biotic and abiotic factors in dynamic floodplain systems, and these factors vary over different spatial and temporal scales (Williner et al. 2010). The regular movements of floodplain decapods are typically active or passive displacements within lakes, ponds or rivers (Williner et al. 2009). Little is known about the dispersal of the large freshwater crab *Zilchiopsis collastinensis* (Pretzmann, 1968). This burrowing crab spends most of its life on river banks in canyons where the locations of caves varies with the river's water level (Williner et al. 2009). More is known about the smaller freshwater crab *Trichodactylus borellianus* Nobili, 1896, which inhabits the roots of water hyacinths and moves passively with macrophyte displacements (Collins et al. 2006).

The dispersal and connectivity of freshwater invertebrate populations are difficult to study directly. One way to perform such studies is by assessing the differences or similarities in the shape of the organisms between populations. Morphometric studies are useful for delineating the shapes of various populations and species over geographical ranges and such studies can provide evidence of regional differences in crustaceans (Rufino et al. 2006, Konan et al. 2010, Silva et al. 2010, Srijaya et al. 2010, Bissaro et al. 2013). Geometric morphometrics (GM) can be used to quantify the variation in these forms (Monteiro 1999), generating a set of shape variables that can be used to test statistical hypotheses and providing a means of visually describing patterns of shape differences in the data (Rohlf and Marcus 1993, Adams 1999). Previous studies have used GM to compare populations of freshwater decapods (Giri and Collins 2004, Giri and Loy 2008, Barría et al. 2011, Idaszkin et al. 2013). The aim of this paper was to study and infer about the population connectivity of *Z. collastinensis* and *T. borellianus* in the context of a floodplain system, through the analysis the morphological variations observed among crabs from localities of the middle Paraná River during two phases of their habitat's hydrological regime.

Methods

Zilchiopsis collastinensis and *T. borellianus* were collected from macrophytes using hand nets and from caves by hand (*Z. collastinensis* only). Samples were collected from sites (localities) along the Paraná River (PR1, PR2, PR3, PR4 and PR5), the Saladillo Stream (SS1 and SS2), the Salado River (SR1 and SR2) and the Coronda River (CR) (Fig. 1). After collection, the specimens were chilled and then preserved with 96% ethanol for further Geometric Morphometrics (GM) analysis. The individuals collected were deposited in the Laboratorio de macrocrustaceos del Instituto Nacional de Limnología (INALI-CONICET-UNL).

Each site was sampled during two different phases of the hydrological regime (i.e., when water levels were falling and rising in the Paraná and Salado River) (Fig. 2). Data on hydrometric levels were obtained from local ports and from the Facultad de Ingeniería y Ciencias Hídricas (Universidad Nacional del Litoral). In addition, conductivity, pH and temperature were measured at each site with a digital sensor (HANNA 198130) (Table 1). The geographical locations according to geographic coordinates of the sampling sites were obtained using GPS tracking (Garmin Dakota 20).

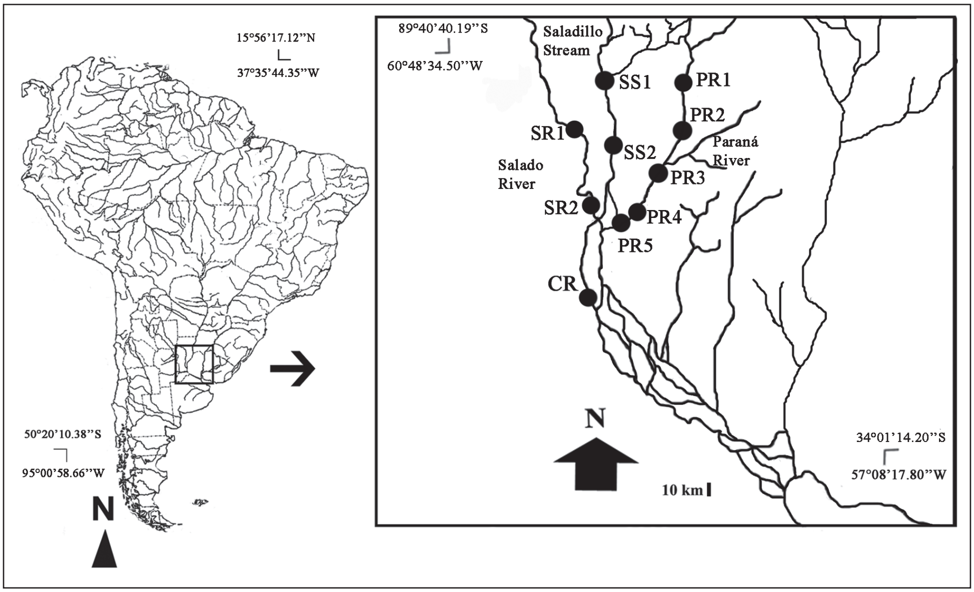

Figure 1. Sampling sites (localities): Paraná River (PR1, PR2, PR3, PR4, PR5); Saladillo Stream (SS1, SS2); Salado River (SR1, SR2); Coronda River (CR).

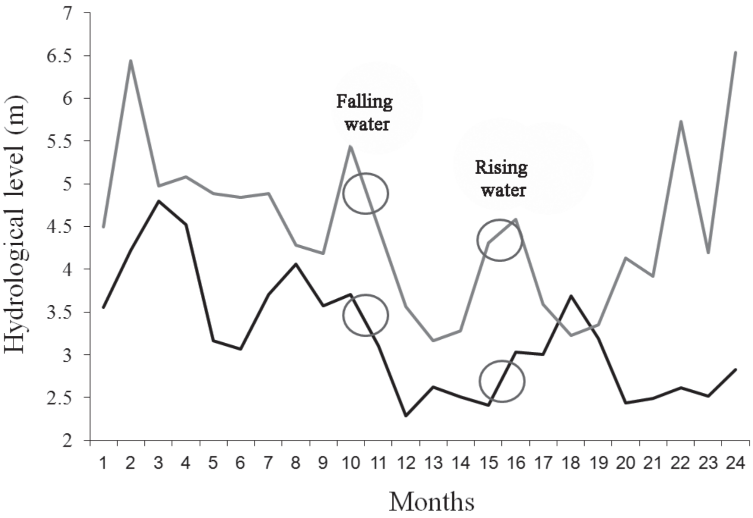

Figure 2. Different phases of the hydrological regime according to hydrological level. Paraná River (black line) and Salado River (gray line). The circles indicate the water level in which the crabs were collected from each river (falling and rising water).

To apply the GM analysis, digital images of each crab's cephalothorax were taken using a Sony Cyber-shot digital camera with a 12.1 mp resolution. The cephalothorax structure of *Z. collastinensis* was represented using 16 digitised landmarks (Type I: LMs #2 to 7; LMs #11 to 16 and Type II: LMs #1, 8, 9 and 10) (Bookstein 1991) (Fig. 3a).

Table 1. Environmental variables measured in each sample site during two hydrological phases of the middle Paraná River basin. Paraná River (PR1, PR2, PR3, PR4 and PR5); Saladillo Stream (SS1, SS2); Salado River (SR1, SR2); Coronda River (CR).

Sampling sites	Falling water			Rising water		
	Conductivity (µS cm^{-1})	Temperature (°C)	pH	Conductivity (µS cm^{-1})	Temperature (°C)	pH
PR1	130	27.4	8.29	130	23.7	7.78
PR2	90	21.2	8.31	120	17.5	8.25
PR3	60	26.8	7.82	130	23.3	8.25
PR4	90	21.6	8.02	-	-	-
PR5	80	22	8.1	-	-	-
SS1	760	26.1	8.02	-	-	-
SS2	-	-	-	1340	20.3	7.82
SR1	-	-	-	2680	21.3	7.9
SR2	1470	24.1	7.87	4680	23.2	7.99
CR	390	25.7	7.94	370	23.7	8.21

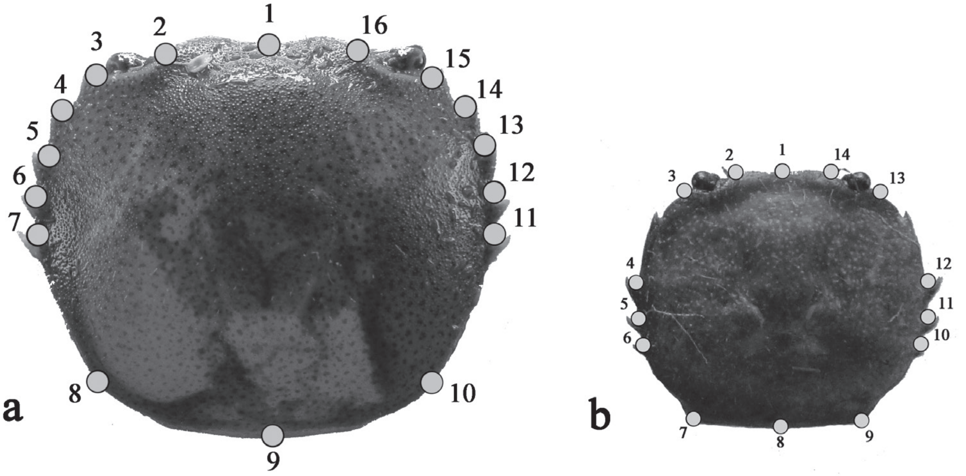

Figure 3. Cephalothorax with configuration of 16 landmarks on *Zilchiopsis collastinensis* (**a**) and 14 landmarks on *Trichodactylus borellianus* (**b**).

For *T. borellianus*, 14 landmarks were digitised (Type I: LMs #2 to 7; LMs #9 to 14 and Type II: LMs #1 and 8) (Fig. 3b). The computer program tpsDig 1.40 was used to digitise these landmarks (Rohlf 2004).

Following the GM analysis, the shape symmetric components associated with position, rotation, translation and size were removed using the Procrustes fit in the program MorphoJ (Klingenberg 2011). Variation in the shape symmetric components was explored via a principal component analysis (PCA) applied to the Procrustes coordinates. Intrapopulation allometry was tested using multivariate regression, with centroid size as the independent variable. The size correction was made by taking into account the residuals of the common pooled within-group regression in the program MorphoJ. Sexual dimorphism was also tested. However, the results ultimately showed that male and female crabs displayed similar variation in shape among localities for both species, and therefore samples were not segregated by sex in subsequent analyses.

Permutations were used to establish the significance of each statistical test, employing 10,000 permutations for the multivariate regression (Klingenberg 2011).

Variations in the shape symmetric component among sites for each moment of the hydrologic regime were analysed using Procrustes pairwise permutation tests and Canonical Variate Analyses (CVA) with the program MorphoJ (10,000 permutations), with residuals of the pooled within-group regression as the focal dataset.

The covariations among shapes, environmental variables and geographical location (altitude and longitude) were analysed with the software tpsPLS (Rohlf 2006) using a permutation test with 99 randomisations. This program uses a two-block partial least-squares analysis, calculating the covariation and correlation between shape and a set of variables (Rohlf 2006). We used this type of analysis to examine whether shape among crabs of different localities varied as a result of certain environmental variables or geographical location in each river during the two phases of the hydrological regime.

Results

The number of specimens collected and analyzed differed for each site depending on the phase of the hydrological regime (Table 2). The geographical locations of the sampling sites were relatively close together (Table 2).

Variation in shape was ordered along PC1 by site at the two hydrological periods for *Z. collastinensis* (PC1: 72.42% and PC2: 10.00% when water levels were falling; PC1: 74.53% and PC2: 6.98% when water levels were rising) and along PC2 by site for *T. borellianus* (PC1: 44.62% and PC2: 15.72% when water levels were falling; PC1: 41.55% and PC2: 16.59% when water levels were rising).

All crabs from all localities of both species exhibited significant ($p < 0.05$) allometric relationships between cephalothorax shape and centroid size during two phases of the hydrologic regime.

Differences in shape variation were observed among the crabs of the Paraná and Salado Rivers during the two phases of the hydrologic regime (Figs 4 and 5) (Procrustes pairwise permutation tests with CVA). For *Z. collastinensis*, individuals of localities in the Paraná River were similar in shape when water levels were falling. Additionally, individuals collected from PR1 had shapes similar to those collected in SR2 (Salado River site) (Fig. 4a) (Table 3) and in CR (Fig. 4a) (Table 3). When water levels were rising, individuals collected from localities in the Paraná River were similar in shape (Fig. 4b) (Table 3). No differences in shape were observed among individuals in localities in both rivers compared to those in CR (Fig. 4b) (Table 3). Individuals collected from both rivers were more similar in shape when water levels were falling than when water levels were rising (Fig. 4a and b). When water levels were rising, crabs collected

Table 2. Number of specimens collected (*Zilchiopsis collastinensis* and *Trichodactylus borellianus*) and analyzed during two different phases of the hydrological regime of the middle Paraná River basin. Paraná River (PR1, PR2, PR3, PR4 and PR5); Saladillo Stream (SS1, SS2); Salado River (SR1, SR2); Coronda River (CR).

Sampling Sites	Geographical location		Falling water		Rising water	
	latitude	**longitude**	***Z. collastinensis***	***T. borellianus***	***Z. collastinensis***	***T. borellianus***
PR1	30°35'01.07"S	59°56'58.31"W	8	-	5	7
PR2	31°10'06.50"S	60°08'16.87"W	-	21	11	-
PR3	31°35'13.51"S	60°33'06.06"W	-	13	8	-
PR4	31°38'33.31"S	60°40'35.83"W	-	20	-	11
PR5	31°39'02.96"S	60°40'29.45"W	20	-	-	-
SS1	30°27'03.45"S	60°05'37.14"W	-	13	-	-
SS2	31°16'44.69"S	60°33'25.10"W	-	-	-	27
SR1	30°59'58.47"S	60°49'48.11"W	-	-	7	-
SR2	31°37'30.11"S	60°45'42.32"W	32	23	-	17
CR	31°43'32.93"S	60°45'22.47"W	6	25	14	12
Total			**66**	**115**	**45**	**74**

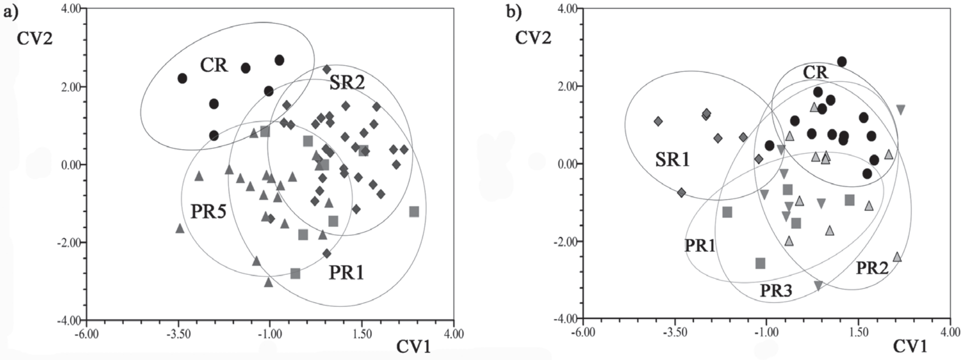

Figure 4. Graphics of Canonical Variate Analyses (CVA) of cephalothorax shapes of *Zilchiopsis collastinensis* between localities. Ellipses represent the confidence interval at 90%. Paraná River (PR1, PR2 PR3, PR5); Salado River (SR1, SR2); Coronda River (CR). **a** falling water **b** rising water.

from localities in the Paraná River had overlapping shapes but these shapes were different from those of crabs collected from the Salado River site (Fig. 4b).

For *T. borellianus*, despite some differences in the results of the CVA for samples collected when water levels were falling, individuals from localities in the Paraná River were similar in shape (Fig. 5a) (Table 3). These were different in shape compared to those in the CR site (Fig. 5a) (Table 3). Shape variation differed when water levels were rising, being the crabs from localities in the Paraná River similar in shape to crabs from CR (Fig. 5b) (Table 3). For both phases of the hydrologic regime, crabs from localities in the Paraná River had overlapping shapes, but those shapes were different from those for crabs in SR2 (Fig. 5a and b).

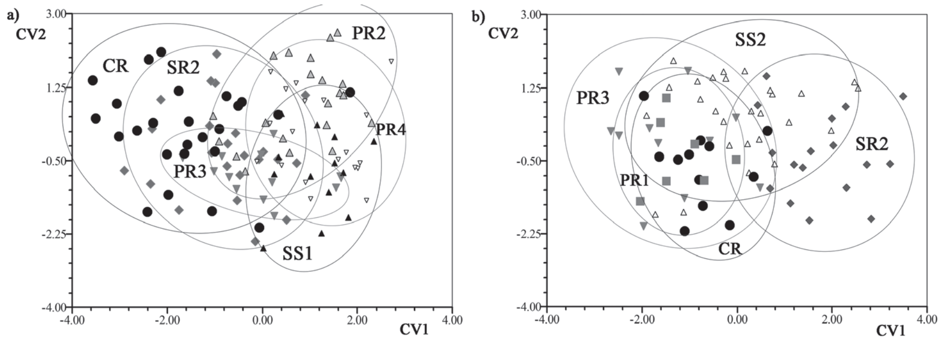

Figure 5. Graphics of Canonical Variate Analyses (CVA) of cephalothorax shapes of *Trichodactylus borellianus* between localities. Ellipses represent the confidence interval at 90%. Paraná River (PR1, PR2 PR3, PR4); Saladillo Stream (SS1, SS2); Salado River (SR2); Coronda River (CR). **a** falling water **b** rising water.

Table 3. Procrustes pairwise permutation tests with Canonical Variate Analyses (CVA) of cephalothorax shapes of *Zilchiopsis collastinensis* and *Trichodactylus borellianus* between localities, during two different phases of the hydrological regime of the middle Paraná River basin. The upper right triangle gives the Procrustes distances and the lower left triangle gives the *p*-values from permutation tests for Procrustes distances among shapes of crabs from localities . Paraná River (PR1, PR2, PR3, PR4 and PR5); Saladillo Stream (SS1, SS2); Salado River (SR1, SR2); Coronda River (CR).

			Z. collastinensis			
Falling water	PR1	PR5	SR2	CR		
PR1	-	0.0104	0.0081	0.0189		
PR5	0.3619	-	0.0107	0.0191		
SR2	0.2779	0.0065**	-	0.0162		
CR	0.0699	0.0402*	0.0069**	-		
Rising water	PR1	PR2	PR3	SR1	CR	
PR1	-	0.0125	0.0115	0.0129	0.0134	
PR2	0.0904	-	0.0143	0.0166	0.0081	
PR3	0.2474	0.0172*	-	0.0131	0.0128	
SR1	0.2887	0.0119*	0.1801	-	0.0128	
CR	0.1262	0.3750	0.0642	0.1193	-	
			T. borellianus			
Falling water	PR2	PR3	PR4	SS1	SR2	CR
PR2	-	0.0158	0.0109	0.0101	0.0127	0.0163
PR3	0.017*	-	0.0185	0.0167	0.0185	0.0173
PR4	0.0771	0.0057*	-	0.007	0.0147	0.0204
SS1	0.1714	0.0178*	0.64	-	0.0147	0.0206
SR2	0.0136*	0.0021**	0.0038**	0.0054*	-	0.0090
CR	0.002**	0.0097*	0.0001***	0.0002***	0.1882	-
Rising water	PR1	PR3	SS2	SR2	CR	
PR1	-	0.0097	0.0133	0.0182	0.0088	
PR3	0.4162	-	0.0146	0.0196	0.0109	
SS2	0.0656	0.0112*	-	0.0098	0.0095	
SR2	0.0006***	<.0001***	0.0645	-	0.0163	
CR	0.4218	0.1778	0.1623	0.0009***	-	

Statistically significant differences, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Table 4. *Zilchiopsis collastinensis* and *Trichodactylus borellianus*: covariations among crab cephalothorax shapes of localities and geographical location during two phases of the hydrological regime of the middle Paraná River basin.

Geographical location	Cephalothorax shape			
	Z. collastinensis		***T. borellianus***	
Falling water	*%Cov.*	*p-value*	*%Cov.*	*p-value*
Dimension 1	0.9954	0.83	0.9898	0.03*
Dimension 2	0.0045	0.18	0.0101	0.98
Rising water	*%Cov.*	*p-value*	*%Cov.*	*p-value*
Dimension 1	0.9835	0.02*	0.9547	0.79
Dimension 2	0.0164	0.99	0.0452	0.22

Statistically significant differences, $^*p < 0.05$.

Zilchiopsis collastinensis and *T. borellianus* presented particular shape variations that were related to geographical location during the two phases of the hydrologic regime. For instance, the covariation between shape and distribution of *Z. collastinensis* when water levels were falling was statistically not significant. In this case, individuals from localities in the Paraná River and the Salado River were more similar in shape, as revealed by the CVA (Fig. 4a) (Table 4). However, significant high covariation between cephalothorax shape and geographical location was observed when water levels were rising (Table 4). Crabs from localities along the Paraná River were ordered by shape according latitude-longitude gradient and this pattern was separate from that of individuals from the site of the Salado River (Fig. 6a). In contrast, *T. borellianus* displayed significant covariation between shape and latitude-longitude when water levels were falling (Table 4), and individual shapes were ordered along a distributional gradient for localities in both rivers (Fig. 6b). Covariation between shape and geographical location was not statistically significant for samples collected when water levels were rising (Table 4). For both species, the crabs of closest sites (based on latitude and longitude) were more similar in shape when covariation was significant (Table 4) (Fig. 6a and b). However, this pattern of covariation was not observed for the two hydrological periods for the two species.

Cephalothorax shape was not significantly related to environmental variables for either species (Table 5), with the exception of when water levels were falling for localities of *Z. collastinensis*. At this time, conductivity was most related to shape (Fig. 7).

Discussion

The relation of cephalothorax shape among localities of two freshwater crabs (*Z. collastinensis* and *T. borellianus*) collected from connected rivers was different during two phases of the rivers' hydrological regimes. In this ecological system, whether water levels were falling or rising impacted the population connectivity for the two species. This suggests that individuals were interchanged among localities by dynamic processes

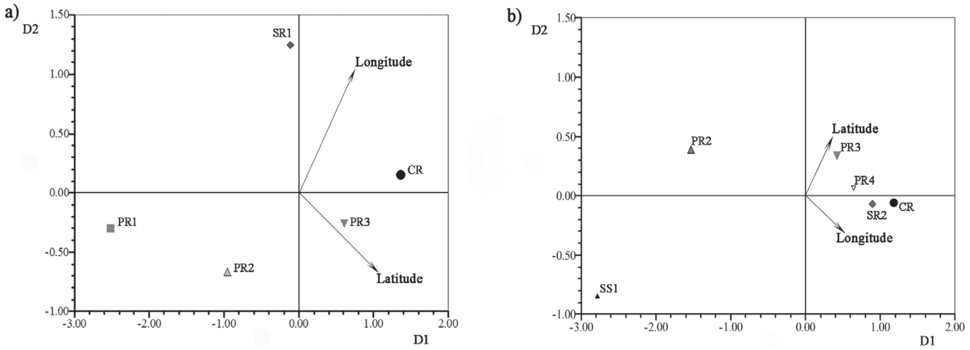

Figure 6. Results of the tpsPLS applying the two-block partial least-squares analysis. **a** *Zilchiopsis collastinensis* when water levels were rising **b** *Trichodactylus borellianus* when water levels were falling. Paraná River (PR1, PR2 PR3, PR4); Saladillo Stream (SS1); Salado River (SR1; SR2); Coronda River (CR).

Table 5. *Zilchiopsis collastinensis* and *Trichodactylus borellianus*: covariations among crab cephalothorax shapes of localities and environmental variables during two phases of the hydrological regime of the middle Paraná River basin.

Environmental variables	Cephalothorax shape			
	Z. collastinensis		*T. borellianus*	
Falling water	*%Cov.*	*p-value*	*%Cov.*	*p-value*
Dimension 1	0.9769	0.01*	0.7321	0.47
Dimension 2	0.9984	0.03*	0.9708	0.20
Rising water	*%Cov.*	*p-value*	*%Cov.*	*p-value*
Dimension 1	0.9020	0.16	0.7050	0.53
Dimension 2	0.9760	0.23	0.9702	0.14

Statistically significant differences, $^{*}p < 0.05$.

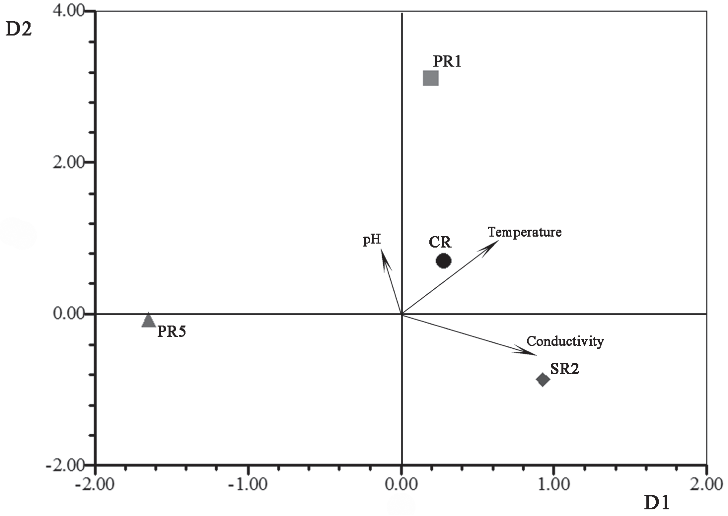

Figure 7. Results of the tpsPLS applying the two-block partial least-squares analysis on *Zilchiopsis collastinensis* when water levels were falling. Paraná River (PR1, PR5); Salado River (SR2); Coronda River (CR).

of the rivers. Generally, rivers in floodplain systems exhibit considerable heterogeneity that varies over multiple temporal and spatial scales (Neiff et al. 2001). Variability over short time scales, such as seasonal flooding, affects the viability of in-stream populations through changes in recruitment, survival and dispersal (Poff et al. 1997). In addition, hydrological connectivity plays an important role in the movement of populations by connecting various landscape patches (Ward 1989, Amoros and Bornette 2002, Pringle 2003).

According to the phases when crabs were more similar in shape (falling water for *Z. collastinensis* and rising water for *T. borellianus*), this can be related to the movements through to a dynamic floodplain system. Generally, water flow patterns become more important in systems with floodplains because currents have an effect on faunal distribution and on the movement of aquatic invertebrates (Olden et al. 2004, Grönroos et al. 2013). As a result, the movements of freshwater decapods are induced by both biotic and abiotic factors in a dynamic floodplain system and these movements can occur over different spatial and temporal scales (Williner et al. 2010). The freshwater crab *T. borellianus* moves passively as a function of macrophyte migrations (Collins et al. 2006). *Zilchiopsis collastinensis* was also found to be associated with macrophytes in this study. Water flow within the hydrological regime is one of the primary factors regulating the growth and distribution of aquatic plants in streams and rivers and affects the passive movements of crustaceans (Chambers et al. 1991). This was also reported by Schiesari et al. (2003), who documented the potential role of macrophyte rafts in the dispersal of organisms across banks and possibly over very large distances in Amazonian rivers.

We did not find a general distribution pattern for crab localities at the two phases of the hydrologic regime. Shapes of *Z. collastinesis* were not related to location during falling water, while shapes of *T. borellianus* were not related to location during rising water. This would imply that the crabs' morphological characteristics were not related to latitude-longitude, with high overlap in shape of crabs among the various localities irrespective the origin of the river. Thus, crabs of even distant localities had similar characteristics in shape. In this sense, the flow of water currents becomes particularly important in floodplain systems because the flow regime organises the river ecosystem and strongly affects population dynamics (Poff et al. 1997, Neiff et al. 2001). Four different density stages were observed for palaemonids and trichodactilids in the Middle Paraná River, coinciding with events in the hydrological cycle (Collins et al. 2006, Williner et al. 2010). Additionally, population increases for the prawn *M. amazonicum* Heller, 1862 in the Amazon River was associated with prawn migrations during floods (Walker and Ferreira 1985). Furthermore, the hydrologic regime of a floodplain system tends to homogenise populations between water bodies during high water periods, attenuating the differences between populations (Gomes et al. 2012). On the other hand, during rising water, the shapes of *Z. collastinensis* were ordered along a distributional gradient according to geographical location. Contrary, shapes of *T. borellianus* were related to latitude-longitude on falling water. During these phases, the crabs of closest sites were the most similar in shape. Similar observations have been reported for *Macrobrachium vollenhovenii* (Herklots, 1851), for which morphological variations between populations were a function of distance between four rivers in Côte d'Ivoire (Konan et al. 2010). Therefore, morphometric analysis proved to be an important tool for evaluating patterns of shape variation for invertebrates by geographical location (Alibert et al. 2001, Krapivka et al. 2007).

Thus, this relationship between shape and latitudinal-longitudinal (distributional) gradient could be affected by the hydrological connectivity between rivers and by the dynamics of the floodplain system. Studies of morphological variation can elucidate patterns observed in phenotypic and genetic characteristics among populations (O'Reilly and Horn 2004). For example, the low morphological and geographical differentiation for the decapod crab *Pachygrapsus marmoratus* (Fabricius, 1787) was attributable to open gene flow and consequent homogenisation (Silva et al. 2009). Additionally, a study of the crab *Carcinus maenas* (Linnaeus, 1758) suggested that there was a high degree of connectivity with little evidence of reduced gene flow (Silva et al. 2010).

In addition, we found some covariation between shape and environmental variables for *Z. collastinensis* during periods of falling water levels. However, the general pattern observed in this study showed that shape was not related to environmental variables for both species of crabs. In floodplain systems, environmental variables are affected by hydrological fluctuations together with hydrological connectivity. These events can regulate population dynamics and constitute an important macrofactor that regulates other environmental variables and can explain the distribution and abundance of organisms that live in these systems (Ward 1989, Neiff et al. 2001). On the other hand, gene flow among populations can counteract gene frequency changes because of selection, imposing a limit on local adaptation. Migration generally has an important role in evolution, affecting spatial patterns and adaptation to local environments (Hellberg et al. 2002).

In this study, we found that the two species demonstrated particular shape variations in relation to geographical location for the two periods in the hydrologic regime. This pattern can be explained by the different behaviours and life histories of each crab. For instance, *Z. collastinensis* is a large burrowing crab that spends most of its life on the banks of rivers in canyons (Williner et al. 2009). *Trichodactylus borellianus* is a small crab that inhabits water hyacinth roots, and this species' movements depend on macrophyte migrations (Collins et al. 2006). This indicates that these crabs could have different dispersal rates, as previously suggested by Bohonak and Jenkins (2003) for invertebrates.

Despite the shape differences found for both crab species during the two periods in the hydrologic regime, shape was more similar for individuals in downstream locality where rivers converge during periods of rising water levels. This suggests that there were exchanges in organisms along the upstream-downstream gradient, referred to as a longitudinal connection (Ward 1989).

These results showed that each freshwater crab species (*Z. collastinensis* and *T. borellianus*) from different localities of the middle Paraná River were connected; however, the flow of organisms changed at different phases of the hydrologic regime. More precisely, this is indicative of a specific type of hydrological connectivity (in an ecological context) that results in the water-mediated transfer of matter, energy and organisms within or between elements of the hydrologic cycle. These connections between crabs of localities can change as a function of the hydrologic regime in a floodplain system. This alteration in connectivity is to be expected because hydrological connectivity operates in longitudinal, lateral, vertical and temporal dimensions (Ward 1989), although the vertical and lateral connections were not evaluated in this study. Furthermore, the distinctiveness of floodplain macrosystems is that the level of water affects the dynamics and the relationships among populations (Poff et al. 1997, Neiff et al. 2001).

Even though this study explored the use of geometric morphometrics at a microgeographical scale, genetic analyses are required to better understand the processes of dispersal and population connectivity of freshwater crabs in this dynamic floodplain system. However, the findings of this study are particularly relevant in the context of ecological flows. When rivers are altered by a human activity, a floodplain's hydrologic dynamics might help to maintain the ecological integrity of decapods, influencing the flow and population connectivity.

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Inferences on mating and sexual systems of two Pacific *Cinetorhynchus* shrimps (Decapoda, Rhynchocinetidae) based on sexual dimorphism in body size and cheliped weaponry

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Abstract

Sexual dimorphism in body size and weaponry was examined in two *Cinetorhynchus* shrimp species in order to formulate hypotheses on their sexual and mating systems. Collections of *C.* sp. A and *C.* sp. B were made in March, 2011 on Coconut Island, Hawaii, by hand dipnetting and minnow traps in coral rubble bottom in shallow water. Although there is overlap in male and female size, some males are much larger than females. The major (pereopod 1) chelipeds of males are significantly larger and longer than those of females. In these two *Cinetorhynchus* species, males and females have third maxillipeds of similar relative size, i.e., those of males are not hypertrophied and probably not used as spear-like weapons as in some other rhynchocinetid (*Rhynchocinetes*) species. Major chelae of males vary with size, changing from typical female-like chelae tipped with black corneous stout setae to subchelate or prehensile appendages in larger males. Puncture wounds or regenerating major chelipeds were observed in 26.1 % of males examined (*N* = 38 including both species). We interpret this evidence on sexual dimorphism as an indication of a temporary male mate guarding or "neighborhoods of dominance" mating system, in which larger dominant robustus males defend females and have greater mating success than smaller males. Fecundity of females increased with female size, as in most caridean species (500–800 in *C.* sp. A; 300–3800 in *C.* sp. B). Based on the sample examined, we conclude that these two species have a gonochoric sexual system (separate sexes) like most but not all other rhynchocinetid species in which the sexual system has been investigated.

Keywords
Fecundity, hermaphroditism, mate guarding, protandry, sexual selection

Introduction

The males of many animal species are larger in size than females and equipped with bigger or more specialized weapons (teeth, horns, claws, glands) used in fighting, competition for and defense of females. These observations were first meticulously documented by Charles Darwin (1871), who postulated that these male features were the result of an evolutionary process he termed "sexual selection". Both female choice (epigamic selection) and intramale competition for females (or resources important to them) may be involved in sexual selection (Emlen and Oring 1977, West-Eberhard 1979, 1983, Thornhill and Alcock 1983, Tobias et al. 2012). Emlen (2008) has made an excellent survey and review of weaponry in a variety of animal taxa, summarizing the main hypotheses about selection pressures involved in their evolution.

Mating systems are behavioral strategies used by individuals of both sexes for finding and securing a mate for successful reproduction (Emlen and Oring 1977, Correa and Thiel 2003a, Bauer 2004). Sexual dimorphism in body size and weaponry in crustaceans, as in most other animal taxa, is highly correlated with mating system. Species in which males possess proportionately larger weaponry and body size than females are characterized by mating systems involving fighting for or defense of females, and/or maintenance of territories or other resources important for female reproductive success (Wickler and Seibt 1981, Grafen and Ridley 1983, Ridley 1983, Christy 1987, Correa and Thiel 2003a, Bauer 2004).

In decapod shrimps, sexual dimorphism in size and weaponry is closely associated with the mating system (Wickler and Seibt 1981, Correa and Thiel 2003a, Bauer 2004). In all penaeoidean and many caridean species, males are smaller than females and often live in mobile aggregations, e.g., the "schooling" species of important commercial fisheries. In these species (contrary to Ridley 1983), reproductive females do not advertise an upcoming molt, the time at which they are receptive to mating (all caridean, many penaeoidean species). When reproductive females of high-density species do molt, the mobile males soon find and mate with them via visual cues or contact chemoreception, as the frequency of contact with females is high. In this promiscuous mating system, termed "pure searching" by Wickler and Seibt (1981), males do not fight for or defend females (Correa and Thiel 2003a, Bauer 2004). Intramale competition takes the form of rapid and efficient searching for females. In other caridean species with "temporary mate guarding" or "neighborhood of dominance" mating systems, males are significantly larger than females and use hypertrophied weapons (major chelipeds, third maxillipeds) to fight for and defend females. In some species, such males are attractive to and sought out by females for mating (references in Correa and Thiel 2003a, Bauer 2004). Some caridean species live in pairs and are termed "monogamous" but their pair-living has been shown to be social monogamy (extended mate guarding) and not necessarily sexual fidelity (Knowlton 1980, Wickler and Seibt 1981, Mathews 2002). In such monogamous species, sexual dimorphism is slight compared to that of mating systems involving male guarding or defense of females (Bauer 2004).

The Rhynchocinetidae is a family of marine caridean shrimps found in subtropical and tropical habitats around the world. The common name "hingebeak shrimp" (McLaughlin et al. 2005) refers to the articulated rostrum, a unique character of the family (Chace 1997, Okuno 1997a,b). The family currently consists of two genera, *Cinetorhynchus* and *Rhynchocinetes*, with 11 and 14 species, respectively (Okuno 1997b, De Grave and Fransen 2011). The biological significance of the marked sexual dimorphism in male size and weaponry of *Rhynchocinetes typus* was first demonstrated by Correa et al. (2000, 2003). In a series of subsequent papers, the links among male size and weaponry, mating system and reproductive success have been extensively explored (Thiel and Correa 2004, Dennenmoser and Thiel 2008). In *R. typus*, large "robustus" males are more successful than smaller more female-like "typus" males in copulating with and inseminating females. Females prefer to copulate with robustus males which, in addition to large size, are equipped with large chelipeds with a prominent setal tuft used for advertising sexual and size condition to conspecifics. Such males use these chelipeds to fight with other males over females. In *R. typus*, the third maxillipeds of larger ("robustus") males are hypertrophied, i.e., proportionately larger than those of females, and they are used as jousting weapons in male-male combat (Correa et al. 2003). These robustus characters develop ontogenetically from a female-like morphology in smaller males with an increase in male age and size (Correa et al. 2000). In another species, *R. brucei*, Thiel et al. (2010) found a similar sexual dimorphism in weaponry although not so well developed as in *R. typus*.

Examination of taxonomic species descriptions and personal observations (J. Okuno) have revealed that males with similar robustus males occur in some but not all rhynchocinetid species. Indeed, observations on *R. uritai* from Japanese waters showed that this species is composed of small males and larger females without any sexual dimorphism in weaponry: male and female chelipeds and third maxillipeds are proportionately similar in size (Bauer and Thiel 2011). Unlike *R. typus* and *R. brucei*, the male mating system of *R. uritai* is not one of "neighborhoods of dominance", but rather appears to be a "pure searching" system. Furthermore, *R. uritai*, unlike the other two species, is a protandric hermaphrodite, changing sex from male to female with increasing size (Bauer and Thiel 2011). Thus, there appears to be considerable variation in sexual dimorphism of body size and weaponry, and possibly sexual system, in the genus *Rhynchocinetes* and thus perhaps in its sister genus *Cinetorhynchus*.

Examination of taxonomic descriptions and personal observations by J. Okuno have revealed similar variation in the genus *Cinetorhynchus*. In this study, we explore this variation in two *Cinetorhynchus* species from Coconut Island, Hawaii, in which males exhibit a robustus-type morphology, i.e., large body size and hypertrophied major chelipeds. We analyze sexual dimorphism in body size and weaponry (major chelipeds, third maxillipeds) in these two *Cinetorhynchus* species and compare it with that previously described for *Rhynchocinetes* species. Morphometric data are used to test hypotheses on intramale competition and mating system in the two *Cinetorhynchus* species studied. We also test, with observations on relevant reproductive characters, the alternative hypotheses of gonochoric (separate sexes) versus protandric (sequential hermaphroditism) sexual systems in these species.

Materials and methods

Two cinetorhynchid species were collected by M. Thiel on Coconut Island, Hawaii (19°43'46"N, 155°04'07"W) on March 16–20, 2011. Collections were made at night of these nocturnal species by dipnetting in shallow (wading) depths along the shore and spotting individuals by the reflection of their eyes under the light of headlamps. A few individuals were also captured with the aid of baited traps in somewhat deeper water (2–5 m). Specimens were preserved in 95% ethanol. Great care had to be taken with preservation and transport of males because their major chelipeds autotomize in life or detach in preservative very easily, and many males detached chelipeds in spite of careful handling.

There appeared to be two similar species, which were initially distinguished on the basis of overall body shape ("stout" and "slender"). Furthermore, from the morphological differences apparent in the structure of abdomen and antennular peduncle, the individuals were divided into two distinct species without doubt. But their available names under nomenclatorial rule have not been concluded. Therefore, in this paper, the "slender" one is referred to as *Cinetorhynchus* sp. A. [near *Cinetorhynchus hendersoni* (Kemp 1925)] and the stout species is termed *Cinetorhynchus* sp. B, closely related to *R. intermedius* Edmondson, 1952, which is regarded as a junior synonym of *C. hendersoni* (see Okuno 1997a). For reasons of manuscript readability, we refer to these two species as *C.* sp. A and *C.* sp. B in the following text.

Morphometric measurements on sexual dimorphism were made using a stereomicroscope with an ocular micrometer. A total of 14 males, 6 females of *C.* sp. A, and 50 males, 14 females of *C.* sp. B were examined, measured, and included in morphometric analyses. Specimens were sexed, using the presence (male) or absence (female) of an appendix masculina on the endopod of the second pleopod. The measure of body size, carapace length (CL), was taken as the chordal distance from the posterior edge of the eye orbit to the middorsal posterior edge of the carapace (e.g., Bauer 1986). As illustrated in similar rhynchocinetid species (Correa et al. 2000, Thiel et al. 2010: Fig. 1b), measurements on weaponry [major (first pereopod) chelipeds and third maxillipeds] were taken. The measure of major cheliped size was its propodal length, i.e., the distance from the proximal end of the propodus to the tip of the propodal finger (Thiel et al. 2010: Fig. 1b). For the third maxillipeds, the length of its distal segment (Thiel et al. 2010: Fig. 1b) and the number of dark corneous spines at its distal end (Thiel et al. 2010: Fig. 2c inset) were measured. Observations were made and recorded on the presence or absence of major cheliped injuries and regeneration.

In the course of measurements on the major chelipeds of males, an ontogenetic change in the chela to a subchela (McLaughlin 1980, Bauer 2004) was observed in which the propodal finger became increasingly reduced in larger males. To test the hypothesis of ontogeny in shape of the major chela in males, the length of the fixed (propodal) finger was measured as the distance from its base (a point corresponding to the basal articulation of the movable finger above) and its tip, and compared to total propodal length, the measure of major chela size.

Measures on two characters associated with incubation of embryos ("breeding dress" in Höglund 1943, Bauer 2004) were taken on both sexes to investigate the possibility of male to female sex change. As figured for *R. uritai* (Bauer and Thiel 2011), these characters were (a) the flange width of the basipod of the second pleopod (as in Bauer 1986) and (b) the height of abdominal pleuron 2, defined as the vertical chordal distance from the ventral edge of the pleuron to the middorsal line of the second abdominal somite.

Embryos were removed from incubating females with stage 1 or 2 embryos (as in Bauer 1991) (N = 2 in *C.* sp. A and N = 7 in *C.* sp. B) and counted. The lesser (*d1*) and greater (*d2*) diameters of five embryos, chosen haphazardly from each brood, were taken and measured, with medians used to calculate embryo volume [volume of oblate spheroid, $V = 1/6\ (\pi^{*}d1^{2}{}^{*}d2)$] (Turner and Lawrence 1979). Only embryos in early developmental stages (stage 1 or 2; see Bauer 1991) were utilized for measures of size and volume. The numbers of embryos (Y) were regressed against X, the body size (CL) of females. Log_{10} transformation was done on the dependent and independent variables whose values were plotted to calculate the slope in order to examine allometry of brood production with the equation "$\log Y = b\ (\log_{10} X) + \log a$", where b is the slope of the regression line and a is the y-intercept. The null hypothesis of isometry is $b = 3$, the slope of isometry for volume measures (brood volume as measured by number of embryos brood^{-1}) plotted against a linear variable (CL) (Gould 1966).

Results

Sexual dimorphism in size and weaponry

Although sample size is limited in our collection, it is apparent that in both species some males are markedly larger than most females (Figs 1–2, 3A–B). The size-frequency distributions of both species appear bimodal but only the intermediate and larger size classes were sampled by the collection (Fig. 1). Individuals of *Cinetorhynchus* sp. B reach a much larger size than those of *C.* sp. A (Fig. 1A–B). Sexual dimorphism in cheliped weaponry is pronounced in both species. Males bear elongated, heavy major first chelipeds (pereopod 1) with elongated propodi in the larger males (Figs 2A–C, 3A, 4A–B). Additionally, chelipeds of larger males end in a wrench-like subchela (Figs 2A–C, 3A). Major chelipeds of females by comparison are small (Figs 2D–E, 3B, 4A–B) and terminate in a typical chela with equal fingers (Fig. 3B–D). In *C.* sp. A, some of the larger males have proportionately much larger chelipeds than the smaller ones (Fig. 4A), while in *C.* sp. B, the growth is more linear although with considerable scatter in the data

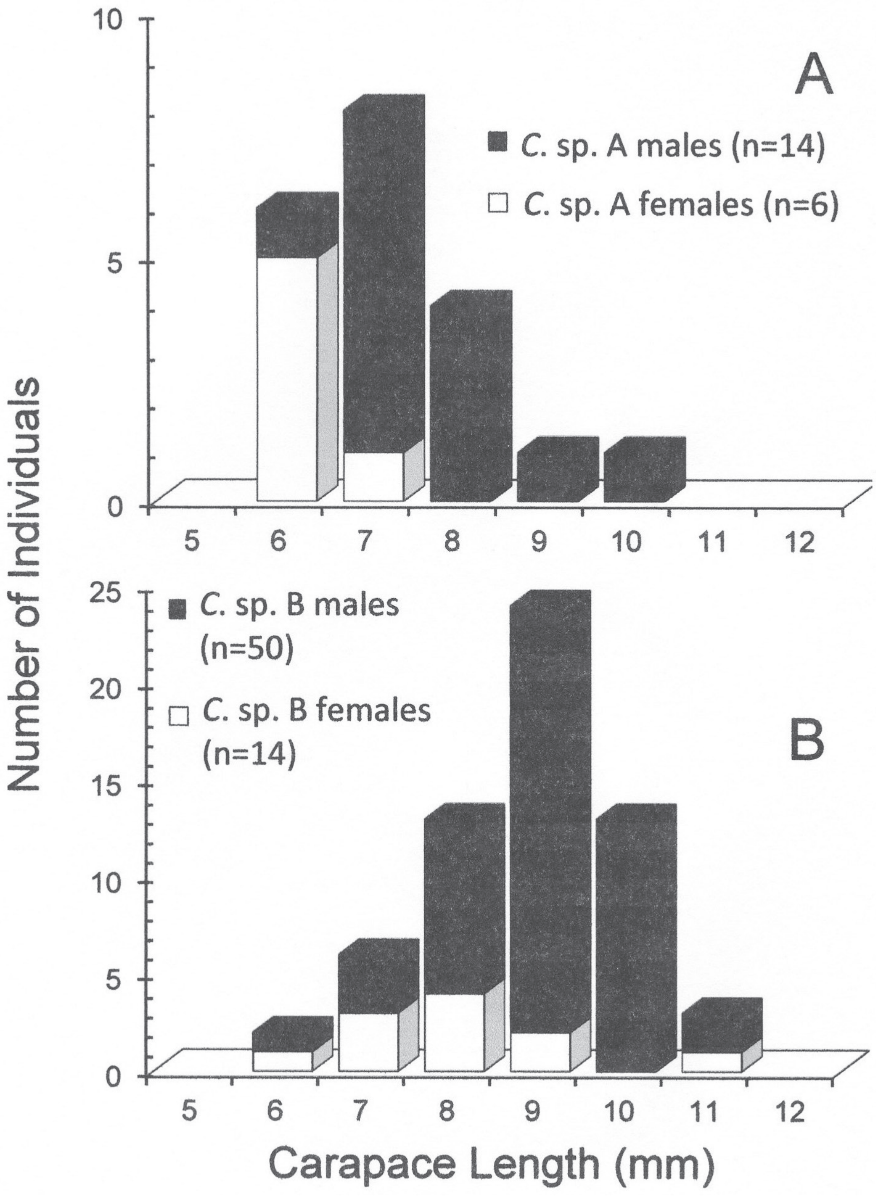

Figure 1. Size frequency diagrams of male and female *Cinetorhynchus* species. **A** *Cinetorhynchus* sp. A **B** *Cinetorhynchus* sp. B.

(Fig. 4B). Female first chelipeds (pereopods 1) are shorter and not markedly robust (Figs 2D–E, 3B, 4A–B). As in all known Rhynchocinetidae (e.g., Holthuis 1993: p. 19, Okuno 1996), the chela fingers of both female pereopods 1-2, as well as male pereopods 2, terminate in several, stout, and black (highly sclerotized) setae (Figs 3C–D, 7A) which are reduced (Fig. 7B–C) or lacking in the major chelipeds of larger males (Fig. 7D–E).

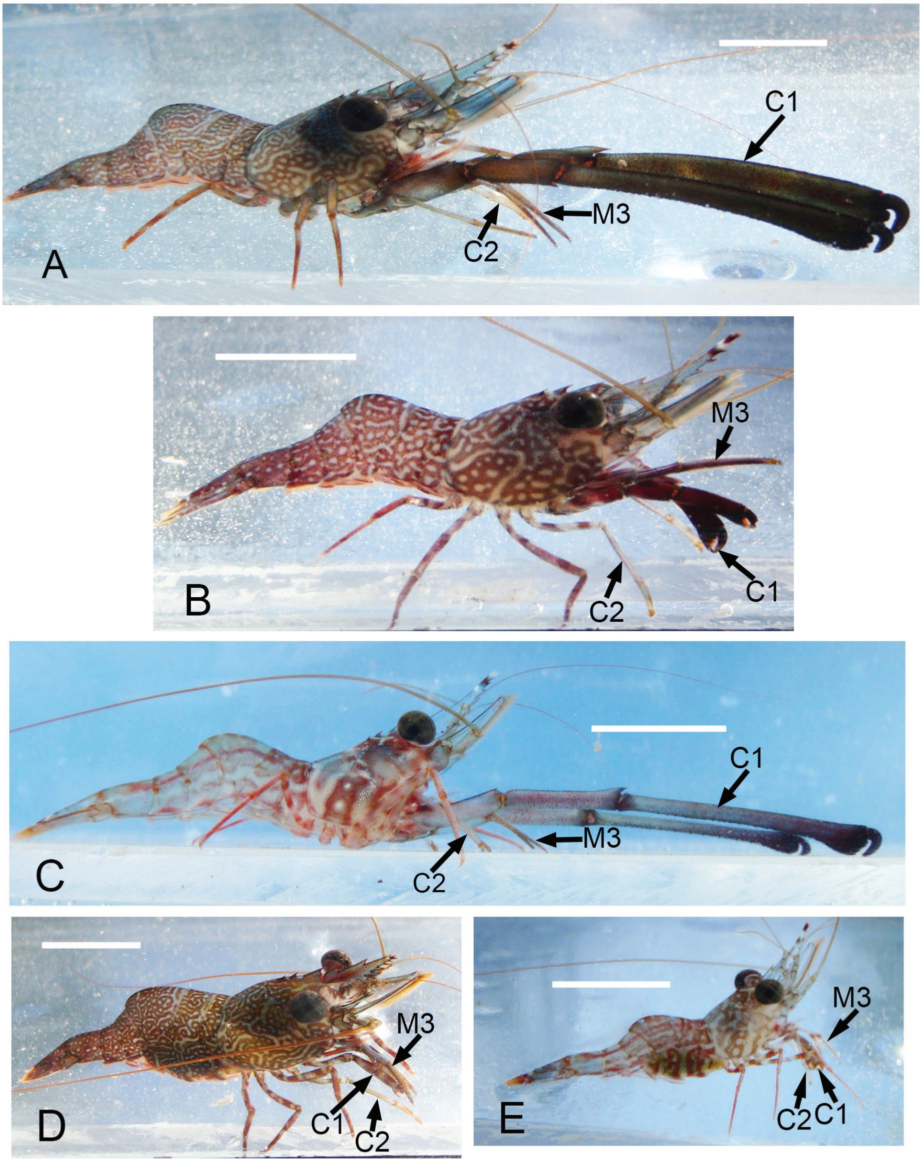

Figure 2. Photographs of living *Cinetorhynchus* species from Coconut Island, Hawaii. **A** *Cinetorhynchus* sp. B male with subchelate first chelipeds (pereopod 1) **B** *Cinetorhynchus* sp. B male with cheliped intermediate between chelate and subchelate **C** *Cinetorhynchus* sp. A male with subchelate chelipeds **D** *Cinetorhynchus* sp. B female **E** *Cinetorhynchus* sp. A female. **C1** cheliped 1; **C2** cheliped 2; **M3** third maxilliped. Scale bars represent 10 mm.

Two standard measures showing possible importance of the third maxillipeds as weapons (Correa et al. 2000, Thiel et al. 2010, Bauer and Thiel 2011) were compared between males and females of both species (Fig. 5A–B). The third maxillipeds of males

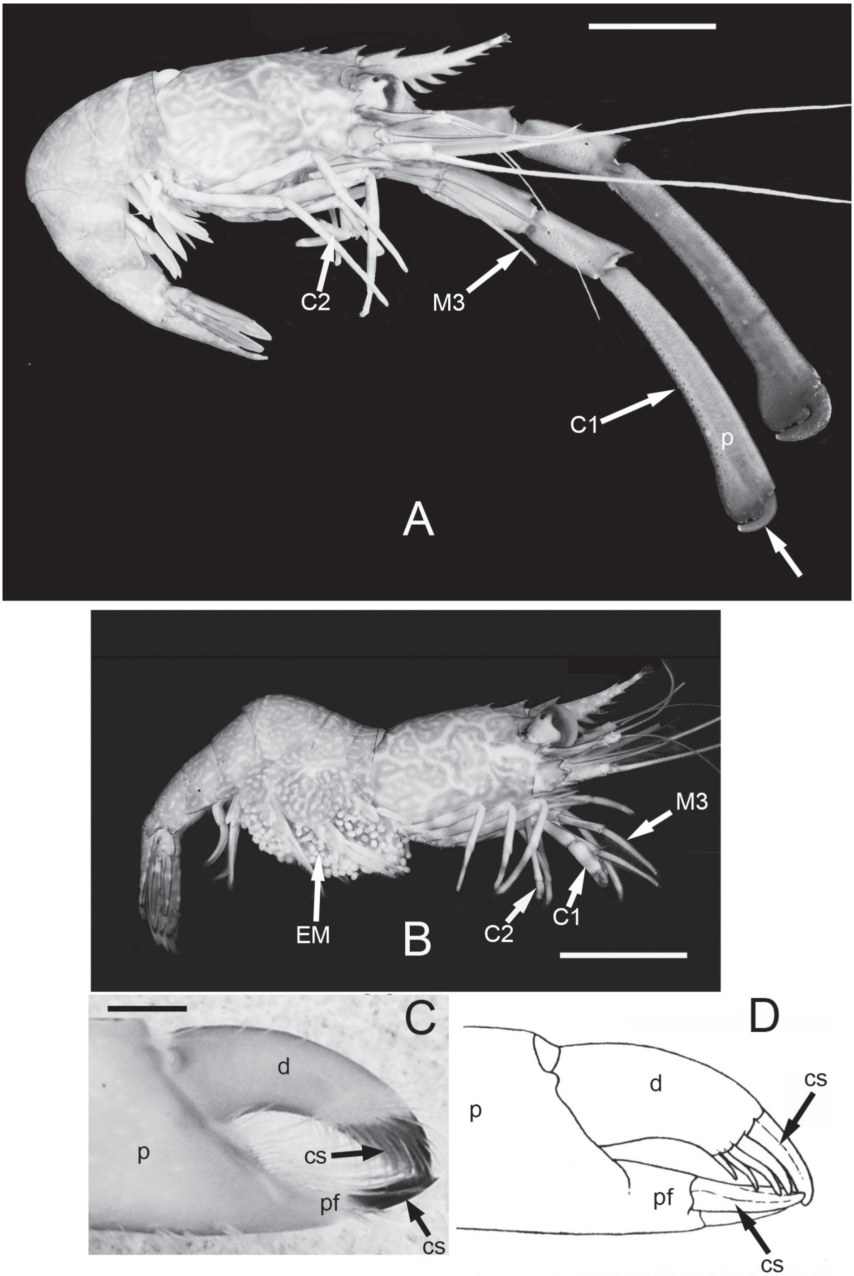

Figure 3. Sexual dimorphism in *C.* sp. B. **A** Large male (10.8 mm CL) **B** Female (8.9 mm CL). Scale bars in A and B represent 10 mm **C** Distal end of chela 1 of a female (11.2 mm CL) showing blackened corneous setae on chela fingers; scale bar represents 0.5 mm **D** Illustration of tip of chela 1, *Rhynchocinetes albatrossae* (from Chace 1997, no scale given), showing form of blackened corneous setae (similar to those shown in Figure 3C) typical of chelipeds 1 and 2 of rhynchocinetids except in large males (e.g., as in this study). **C1** cheliped 1; **cs** corneous setae; **dactyl** (movable finger); **M3** third maxilliped; **p** propodus; **pf** propodal (fixed) finger.

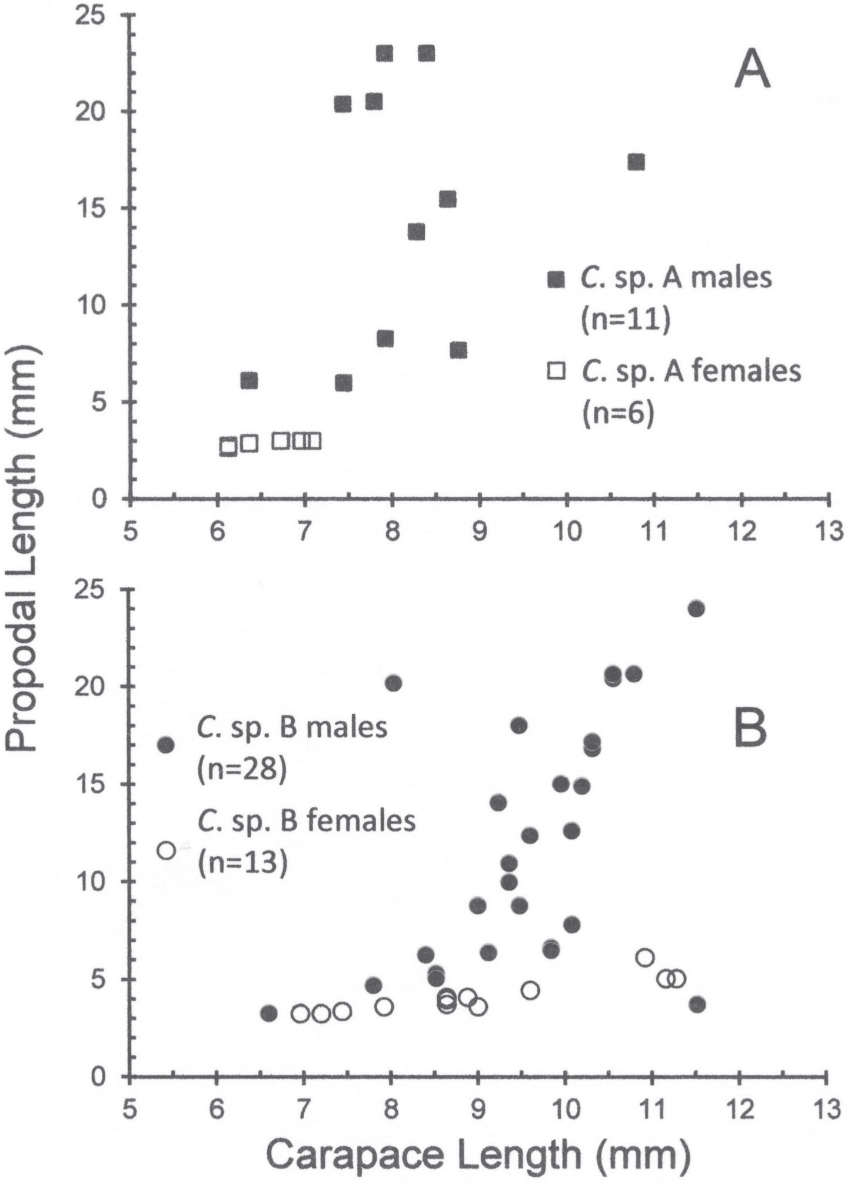

Figure 4. Variation of cheliped size in *Cinetorhynchus* species. Cheliped size (measured as propodal length) is plotted against body size (CL) in male and females. **A** *Cinetorhynchus* sp. A **B** *Cinetorhynchus* sp. B.

in *C.* sp. B (Figs 2A–B, 3A, 5A) are proportionately but not markedly larger than those of females (Figs 2D–E, 3B,5A), which is also the case in *C.* sp. A (Figs 2C,E, 5A), although the sample size of females in the latter is small (N = 6). In both species, there is considerable overlap between the sexes in the number of maxilliped 3 terminal spines (Fig. 5B).

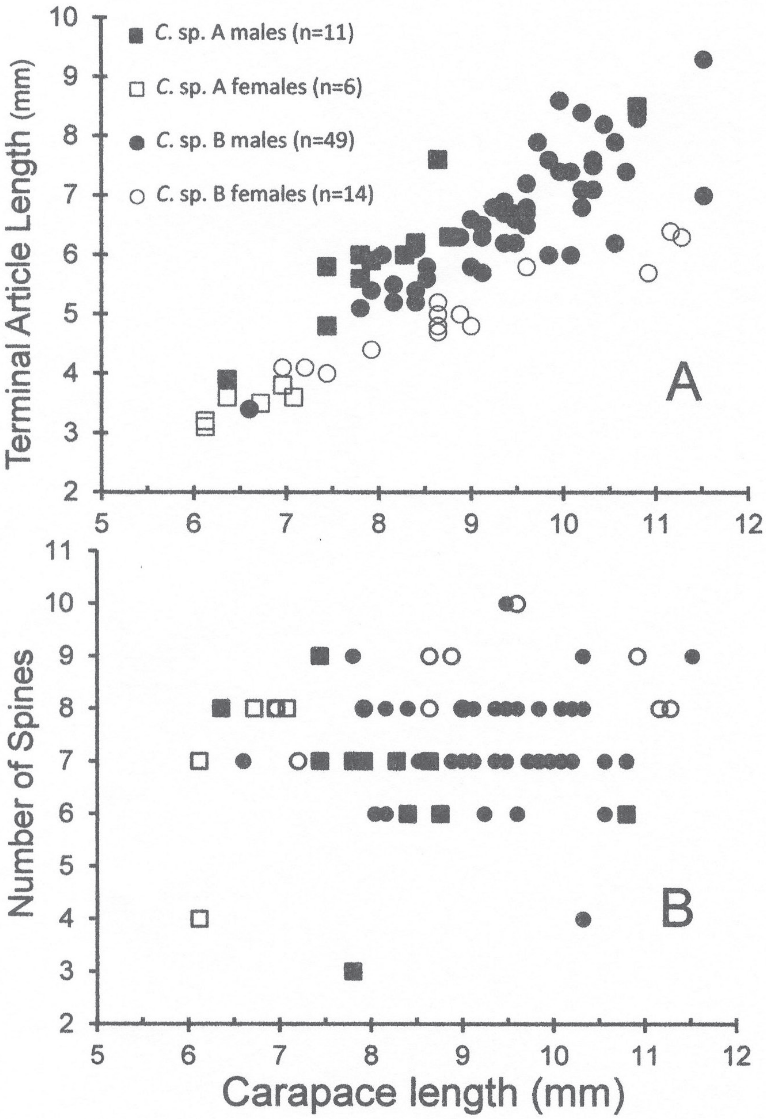

Figure 5. Comparison of third maxillipeds in male and female *Cinetorhynchus* species. **A** Maxilliped 3 size (measured as length of terminal article) plotted against body size (carapace length, CL) **B** Number of corneous spines on the terminal article of maxilliped 3 plotted against body size (CL). In **B** number of observations is the same as **A**, except for *C.* sp. B males (n=45) and *C.* sp. B females (n=13) because spinous portions of the terminal article of some individuals were damaged.

Ontogeny of major chelipeds in males

With increasing size, males of both species show a major change in the shape of the chelae of the major cheliped (pereopod 1) while females do not (Fig. 6A–B). These changes were quantified by comparison of the chela's propodal finger length vs cheliped 1 size, as measured herein by total propodal length (Fig. 6A–B). In males, there is a gradual change

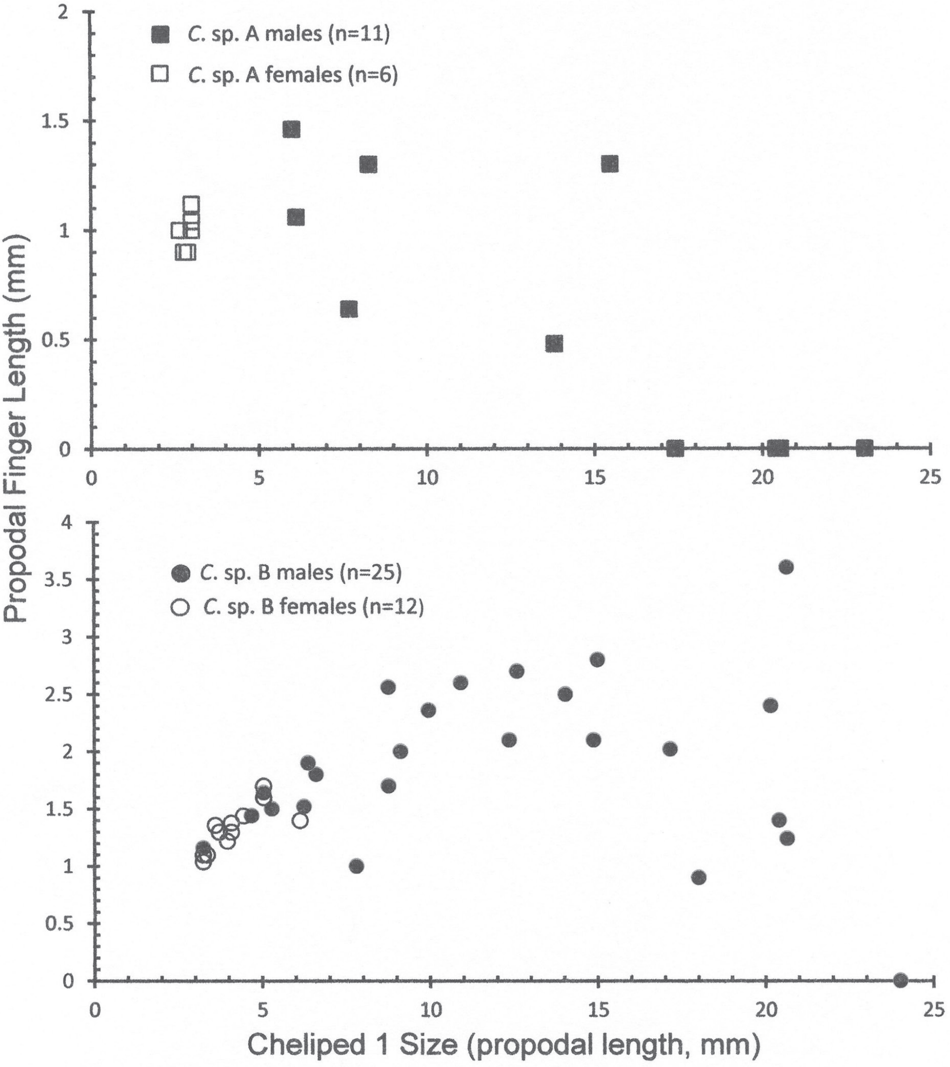

Figure 6. Ontogeny of major chela (pereopod 1) structure from a typical chela to a subchela in *Cinetorhynchus* species A and B. The length of the propodal finger relative to propodal length (cheliped size) is plotted as the measure of chela structure. With growth, the relative propodal (fixed) finger length decreases in larger males but not in females as male first chelipeds change from chelate to subchelate (see Figure 7). **A** *Cinetorhynchus* sp. A **B** *Cinetorhynchus* sp. B.

in the shape of the major chela from a true chela (with distinct upper movable dactylar and lower fixed propodal fingers that close together as in females, Figs 3B–D, 7A) to a more prehensile chela (Fig. 7B–D) to a subchela (Fig. 7E) in the largest robustus-type males. In females, chela 1 has equal propodal and dactylar fingers of a typical chela (Figs 3C–D, 7A), and is tipped with several black sclerotized (= corneus) setae (Figs 3C–D, 7A). In intermediate-sized males, the propodal finger is somewhat reduced compared to

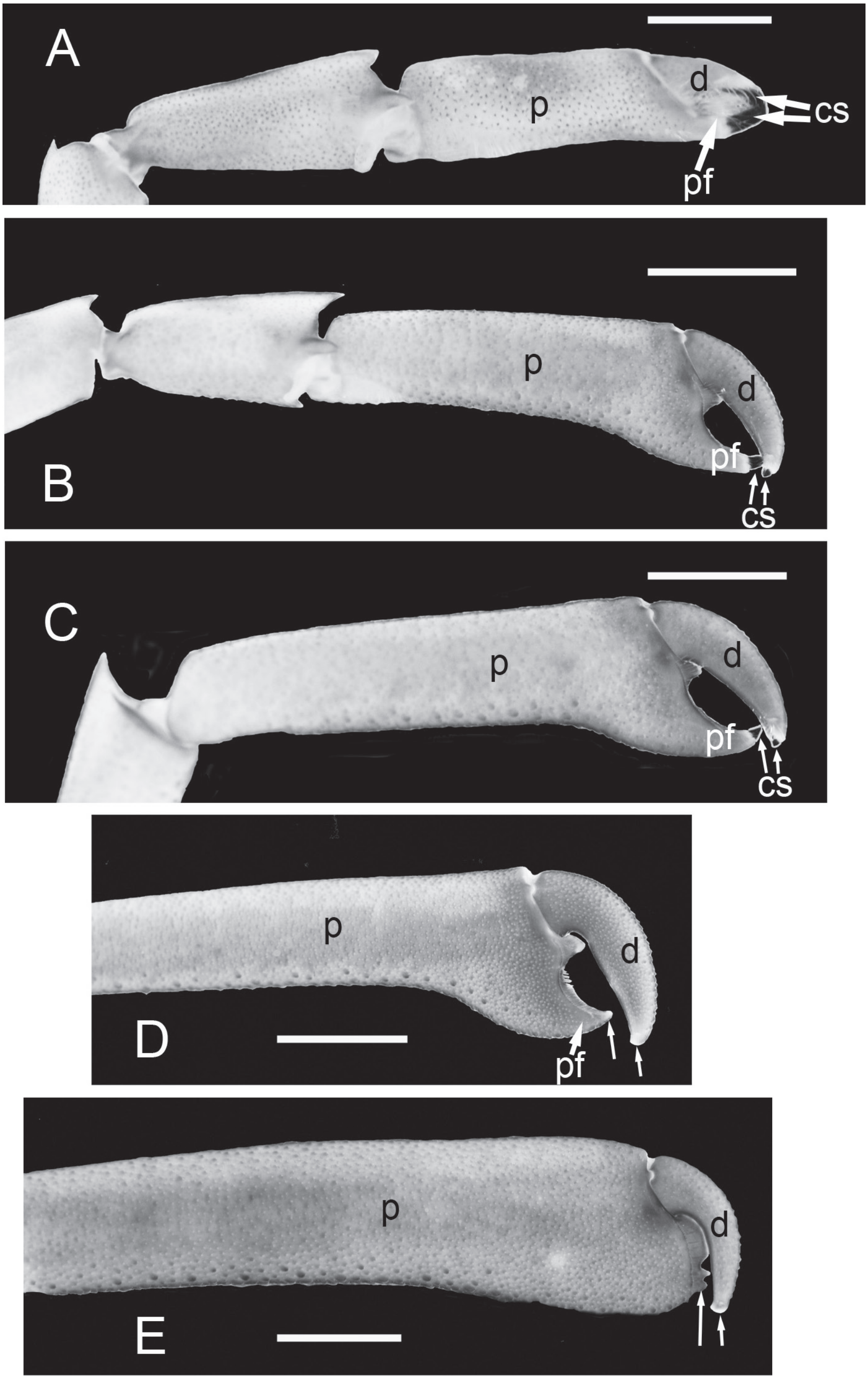

Figure 7. Variation in the major chela and chela finger shape with increasing size in males of *Cinetorhynchus* species B. **A** female 11.3 mm CL **B** male 9.4 mm CL **C** male 10.1 mm CL **D** male 10.6 mm CL **E** male 11.4 mm CL. **cs** corneous (black) seta(e); **d** dactyl (movable finger); **p** propodus; **pf** propodal finger. Unlabeled arrows in **D** and **E** show lack of the black corneous setae seen in **A**–**C**. Scale bars represent 10 mm.

the dactylar finger (Figs 6A–B, 7B–D), and the setation becomes reduced in both fingers to a single black seta at the tip (Fig. 7B–C), which is lost with increasing size (Fig. 7D–E). In addition to decreasing propodal finger size with increasing chela 1 size, the distal end of the propodus also increases in height (Fig. 7B–E). The dactylar finger is flexed (Fig. 7E) against the expanded distal end of the propodus instead of closing against with a propodal finger as in females (Figs 3C–D, 7A), thus forming a subchela.

Injuries to major chelipeds

The major chelipeds of a number of males showed injuries. These injuries took the form of puncture wounds (Fig. 8A–C) to the elongated propodal portion ("palm") of the major chelipeds. Previous possible injury, i.e., complete loss, of the cheliped may be indicated by regeneration of the cheliped (Fig. 8D). Puncture wounds were recognized by roughly circular perforations in the cuticle surrounded by melanized exoskeleton, a sign of scabbing and healing. Regenerating chelipeds were recognized by a combination of characteristics: much smaller than the other member of the pair (if present), a poorly sclerotized (whitish in preservative) cuticle, and a reduced number of poorly formed articles (Figure 8D). Of 38 males (both species) with one or both chelipeds, 10 individuals (26%) showed signs of injury (5 with puncture wounds, 5 with regenerating cheliped). By species, *C.* sp. B males (N = 27), 3 (11.1%) had puncture wounds, while 4 (14.8%) had a regenerating major cheliped. In *C.* sp. A males (N = 11), 2 (18.2%) showed puncture wounds and 1 (9.1%) had a regenerating cheliped. None of the females (*C.* sp. A, *N* = 6; *C.* sp. B, *N* = 14) showed any obvious sign of injury to their major chelipeds.

Fecundity

Cinetorhynchus sp. A had 484–798 embryos ($\bar{x}$ = 601 ±128, *N* = 6), while females of *C.* sp. B carried from 304 to 3786 embryos per brood, ($\bar{x}$ = 1491 ± 104, *N* = 13). Linear regressions on $\log_{10}$-transformed variables (Fig. 9) indicated positive allometry in brood size ($b > 3$) in *C.* sp. B ($b = 3.983$; 95% c.l.: 3.013–4.952). In *C.* sp. A, $b =$ 2.471, a number which could indicate negative allometry as $b < 3$. However, the 95% limits on *b* in *C.* sp. A are so broad (-0.1469–5.090), probably due to small sample size (*N* = 6), that a definite statement on allometry in this species cannot be made. Females of *C.* sp. A had slightly smaller embryos than females of *C.* sp. B (Table 1) but the sample sizes are too small for a meaningful statistical comparison.

Breeding-dress characters and sexual system

To detect possible male to female sex change, two female breeding-dress characters were measured and compared between males and females: the height of the second pleuron

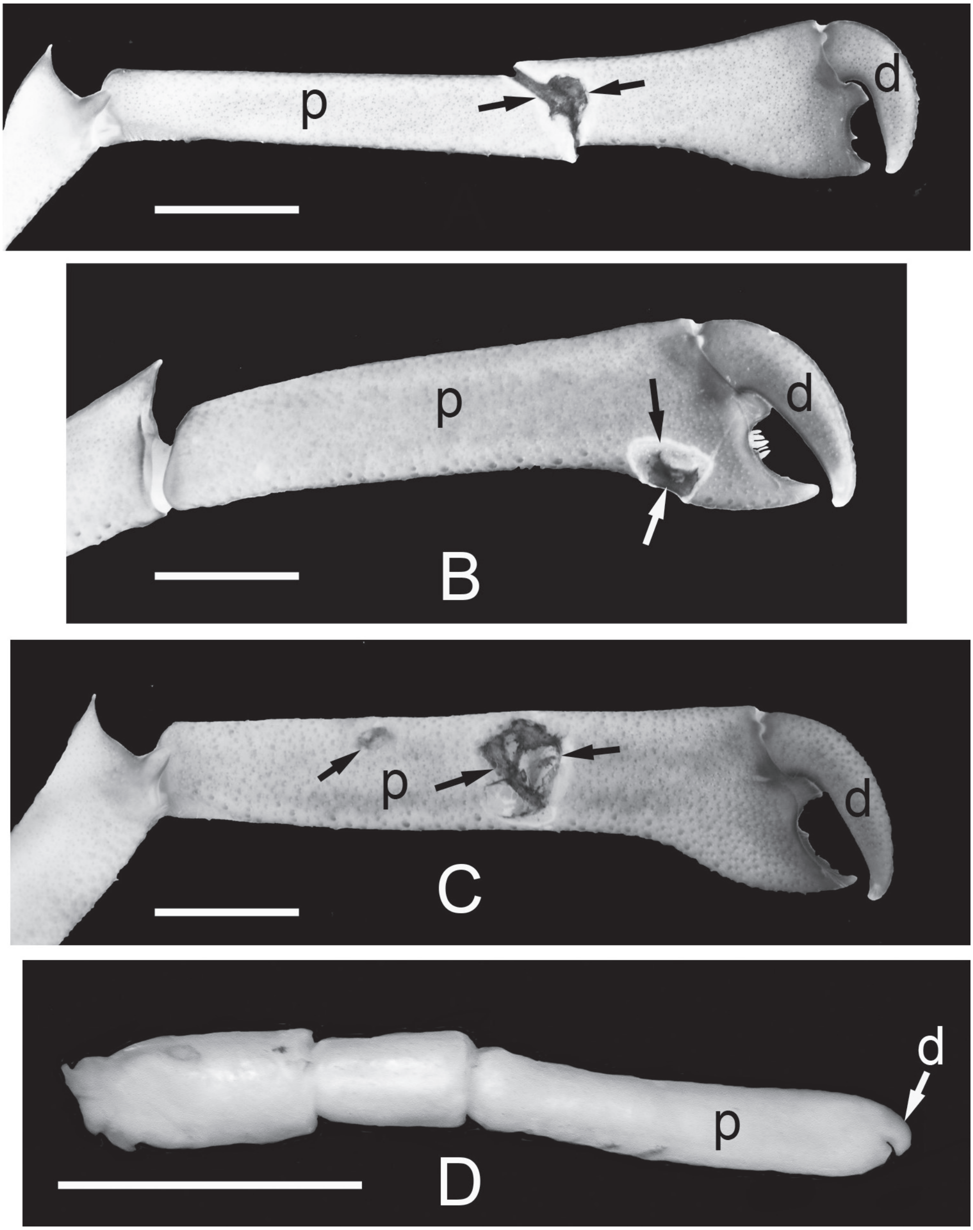

Figure 8. Puncture wounds (unmarked arrows) on the propodi of the major chelipeds of three large *Cinetorhynchus* males. **A** *Cinetorhynchus* sp. A **B** and **C** *Cinetorhynchus* sp. B **D** Regenerating major cheliped of a male *Cinetorhynchus* sp. B; only two articles plus a rudimentary cheliped with underdeveloped propodus and dactyl have formed. **d** dactyl (movable finger); **p** propodus. Scale bars represent 3 mm.

(Fig. 10A) and the width of the basipod flange of the second pleopod (Fig. 10B). In both species the second pleuron is higher and the flange is wider in females than in males (Fig. 10A–B) throughout their size range, typical of gonochoric caridean species.

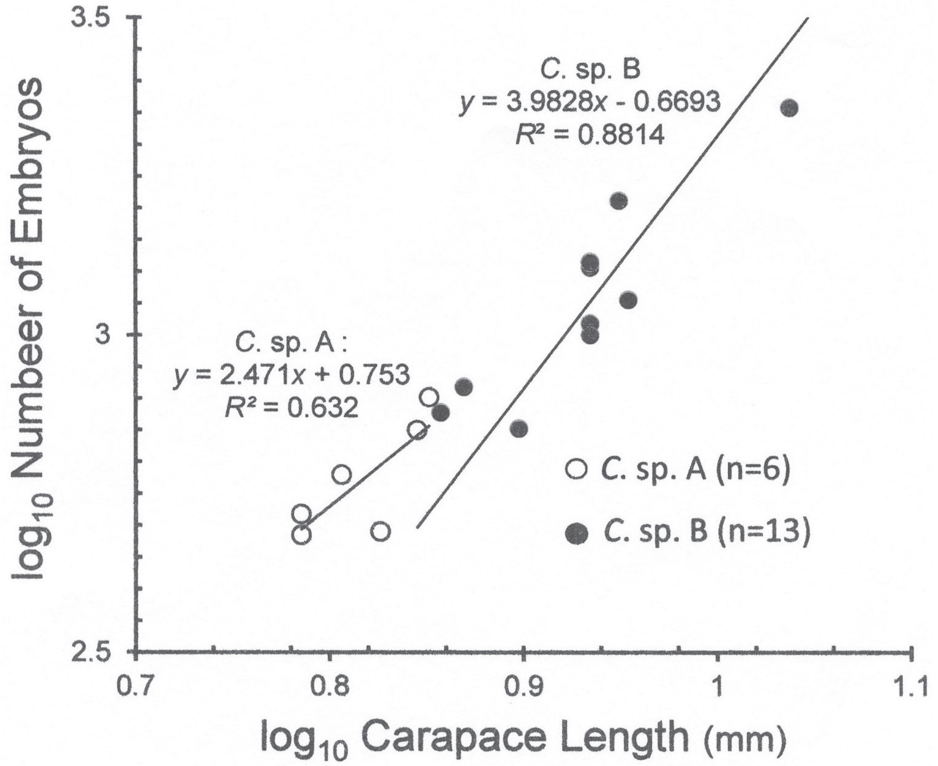

Figure 9. Fecundity in *Cinetorhynchus* species A and B. The $\log_{10}$ number of embryos per brood are plotted against $\log_{10}$ female size (carapace length, mm).

Table 1. Embryo size ($\bar{x}$ ± SD) of *Cinetorhynchus* species from Coconut Island, Hawaii, of females with stage 1 or 2 embryonic development. The medians of the lesser (*d1*) and greater (*d2*) diameters of 5 embryos brood^{-1}, given below, were used to calculate embryo volume.

Species	*d1* (lesser diameter, mm)	*d2* (greater diameter, mm)	Volume (mm^3)	N
Cinetorhynchus sp. A	0.37 ± 0.04	0.47 ± 0.07	0.034 ± 0.013	2 females
Cinetorhynchus sp. B	0.41 ± 0.02	0.50 ± 0.05	0.044 ± 0.007	7 females

Discussion

Sexual dimorphism in size and weaponry

The sexual dimorphism in size and weaponry of the two Pacific species studied allows, for the first time, the formulation of hypotheses about the sexual and mating systems in the genus *Cinetorhynchus*. The population structure, composed of smaller females and larger males in the two *Cinetorhynchus* species, along with the presence of "robustus" males, i.e., large males with hypertrophied appendage weaponry, strongly suggests that the mating system involves some type of male-mate guarding. This type of mating system might vary from temporary (short-term) to extended (socially monogamous)

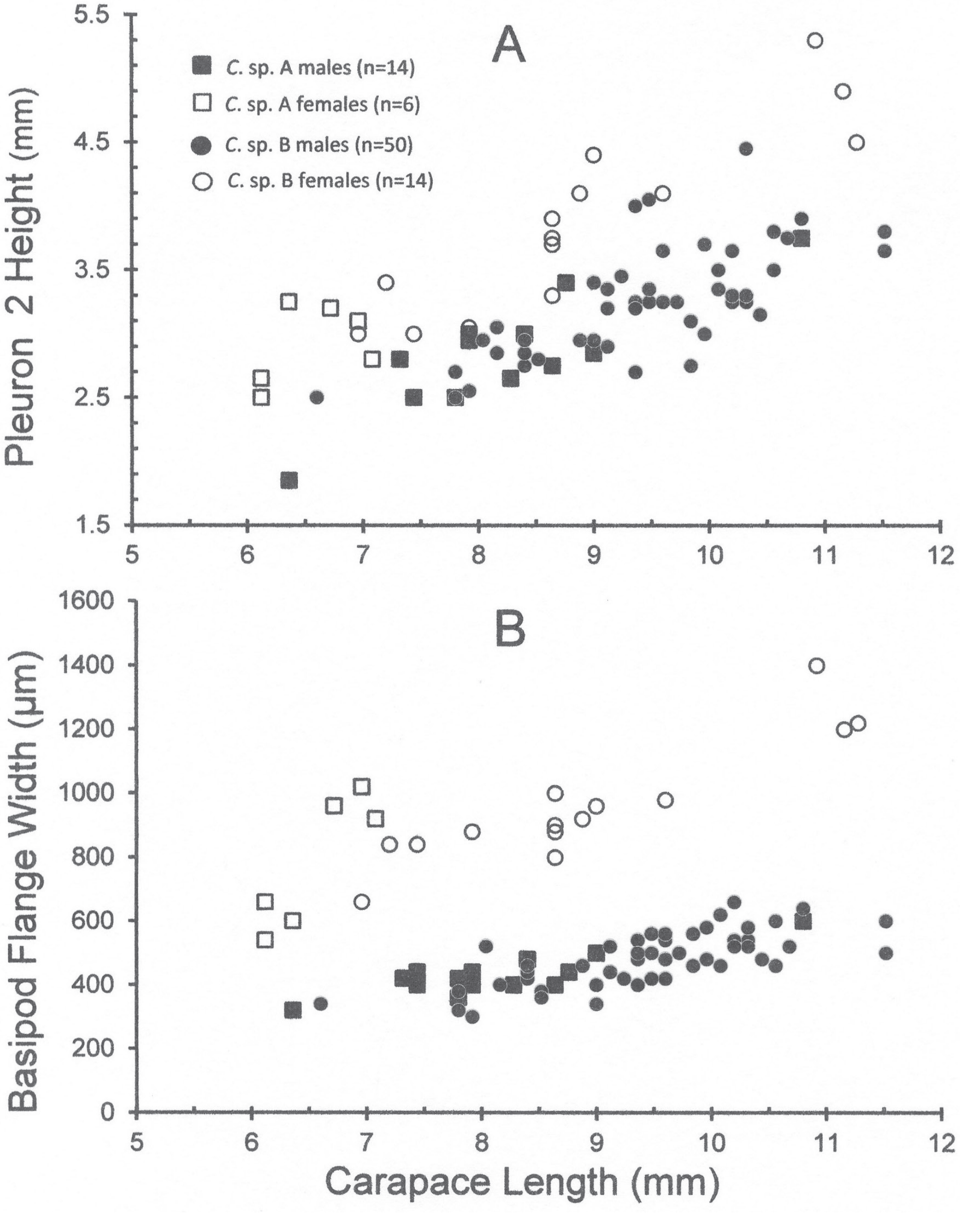

Figure 10. Variation in female breeding dress characters with increasing size in females in *Cinetorhynchus* sp. A and *Cinetorhynchus* sp. B. compared with the same structures in males. **A** Height of the second abdominal pleuron **B** Width of the basipod flange of the second pleopod.

mate guarding (Wickler and Seibt 1981, Bauer 2004) to perhaps the “neighborhoods of dominance” mating system found in species such as *R. typus* and *Macrobrachium rosenbergii* (Correa and Thiel 2003a and references therein).

Ontogeny of major chelipeds in males

The male weaponry of the *Cinetorhynchus* species studied consists of elongated, hypertrophied major (first pereopod) chelipeds bearing chelae whose form in larger males is more like a subchela than a chela. In a subchela, the movable (dactylar) finger folds against a vertically expanded distal end of the propodus (Bauer 2004). Males of at least 3 of 11 described *Cinetorhynchus* species, *C. hawaiiensis* (see Okuno and Hoover 1998), *C. hendersoni* and *C. reticulatus* (see Okuno 1997a) also have hypertrophied major chelipeds. In females, the first chela is a "true" chela, i.e., the movable finger closes against a fixed (propodal) finger of similar length as in all rhynchocinetid species, which might be useful in autogrooming or grazing epibiota of hard surfaces. *Rhynchocinetes typus* is a known grazer and predator on fouling organisms of rocky and other surfaces (Dumont et al. 2011a,b). In males of the two *Cinetorhynchus* species, the second chelipeds are much like those of the females. In smaller males, the first chelae are somewhat female-like, but with increasing cheliped size, the sclerotized setae at the tip are lost, the distal end of the propodus expands vertically. The fixed (propodal) finger becomes proportionately shorter with an increase in cheliped size, so that in the larger "robustus" males, the first chelipeds bear a typical subchela. The loss of the possible grooming/grazing setae of the first chelipeds of robustus males may represent a cost associated with increased fighting ability. The considerable variation in cheliped-1 finger size in larger males might be due to loss of the cheliped in fighting and thus represents variation in the stage of regeneration and subsequent growth of the cheliped.

Injuries to major chelipeds

The size and form of the male first chelipeds, as well as injuries to the first chelipeds (puncture wounds on the chela 1 propodus, regenerating chelipeds) in some males clearly suggests fighting between males. Presumably, such combat would be for access to or defense of females or resources attractive to them, as in many animals with hypertrophied weaponry (Emlen and Oring 1977, Emlen 2008). Rypien and Palmer (2007) found puncture wounds on the major chelipeds of a porcellanid crab, but these injuries occurred in both sexes and were more frequent in high density populations in wave-swept habitats than in low density and protected sites. The authors interpreted these data as evidence of both abiotic physical injuries and fighting among individuals of both sexes. Our observations on major male chelipeds injuries in the two *Cinetorhynchus* species are very similar to those of Rojas et al. (2012), who observed puncture wounds on the major chelae of males in the river shrimp *Cryphiops caementarius* (Palaemonidae) and reviewed examples from other decapods in which injuries are attributed to intramale fighting. Similar observations on damage in large males of *R. brucei* were also interpreted as being caused by intramale fighting (Thiel et al. 2010). In the *Cinetorhynchus* species studied, we found no puncture wounds nor regenerating chelipeds in females, supporting the hypothesis that male injuries were a result of intramale combat. The hypothesis that the puncture wounds and regenerating first chelipeds observed result from fighting and not some other form of injury (predators, or physical injury by, for example, wave action in a coral-rubble habitat on elongated and exposed appendages) requires testing.

Hypertrophied weaponry is usually assumed and often has been shown to have evolved in many animals either because of intrasexual competition (fighting ability) among males for females and/or by female selection of males as potential mates based on their size and weaponry or other attractive characteristics (Darwin 1871, Emlen 2008). In *R. typus,* robustus males do assess the fighting potential of other males when competing for females (Correa et al. 2003). In this species, males display their major chelipeds in the presence of receptive males by raising and lowering the major chelipeds. Furthermore, females are attracted to and chose among male suitors based on sexually dimorphic characters and behavior (Diaz and Thiel 2003, 2004). Sexual dimorphism in body size and weaponry in the two *Cinetorhynchus* species studied herein may similarly function in female selection of males.

The form of the subchela and the types of injuries found in the *Cinetorhynchus* species studied suggests the hypothesis that males may use their major chelae to grasp the long propodus of their opponent's chelae, perhaps flipping or pushing them away from the female or refuge in which they are found. In *R. typus*, if cheliped displays between similarly matched robustus males fail to resolve a contest over a receptive female, males fight by grasping each other's major chelae and jabbing at each other with hypertrophied and spear-like third maxillipeds, often injuring each other (Correa et al. 2003). Although the third maxillipeds of the *Cinetorhynchus* species in this study were proportionately slightly larger than in the females, these appendages were similar in size and appeareance between the sexes, i.e., typical carideans third maxillipeds (see Bauer 2004). There was not the pronounced sexual dimorphism found in *R. typus* and *R. brucei* (Correa et al. 2000, Thiel et al. 2010). We propose that third maxillipeds of males in the two studied *Cinetorhynchus* species are not significant weapons as in *R. typus* and *R. brucei*. Such hypotheses in these two *Cinetorhynchus* species must await evaluation of larger samples over a longer temporal period, and especially for observations and experiments on living shrimps.

Fecundity

Fecundity comparisons of these two *Cinetorhynchus* species with species of *Rhynchocinetes* (*R. uritai* and *R. typus*) suggest that fecundity is similar when body size differences are taken into account (Bauer and Thiel 2011, Vásquez and Castilla 1982, respectively). Our limited results suggest positive allometry in brood size (number of embryos with female size) in *C.* sp. B and perhaps negative allometry in *C.* sp. A. Early stage embryos were twice as large in size (an order of magnitude larger in embryo volume) than those reported for *R. uritai*. Differences in embryo size and volume may indicate different fecundity/incubation period/larval development strategies among rhynchocinetid species that warrant further and more comprehensive study.

Breeding-dress characters and sexual system

Males of these two species are as large or larger than females, a size frequency distribution which does not indicate protandric (male to female) sex change. However, given the finding of protandry in another rhynchocinetid species, *R. uritai* (Bauer and Thiel 2011), other morphological characters indicating male to female sex change were analyzed. Key characters that indicate sex change are those involved in the reproductive female spawning/incubation chamber below the abdomen, i.e., the "breeding dress" (Höglund 1943). The height of the second pleuron and the width of the basipod flange of the second pleopod are two such characters (Bauer 1986, Bauer and Thiel 2011). In the *Cinetorhynchus* species examined here, there was no morphological shift in males to a female-like condition with an increase in size as one would expect in a sex-changing species. Thus, transitional individuals (sex changers) of intermediate external sexual morphology were not observed. The evidence presented here shows that these two *Cinetorhynchus* species, with large males bearing hypertrophied chelipeds, are gonochoric (separate sexes), as in two other such "large male" species investigated, *R. typus* (Correa and Thiel 2003b) and *R. brucei* (Thiel et al. 2010).

In both *Rhynchocinetes* (e.g., *R. typus*, *R. brucei*) and *Cinetorhynchus* (this study), the two current genera of the Rhynchocinetidae, there are species with large male size and hypertrophied male weaponry. A preliminary survey of the taxonomic literature on rhynchocinetids (J. Okuno, pers. obs.) indicates that there are also a number of species in the family in which males are on average smaller than females and without hypertrophied weaponry (authors pers. obs.). At least one species, *R. uritai*, has a protandric sexual system (Bauer and Thiel 2011). Thus, this family may serve as an excellent model for testing hypotheses on the evolution of sexual and mating systems as well as sexual selection. The costs (e.g., higher exposure to injuries by predation, intramale combat, physical injury, energetic costs of growth) and benefits (access and/or attractiveness to potential mates) of hypertrophied sexual weaponry have been and continue to be a topic of considerable interest to evolutionary biologists (e.g., Darwin 1871, West-Eberhard 1979, 1983, Emlen 1983, Emlen 2008, Tobias et al. 2012). We suggest the family Rhynchocinetidae is an excellent system for such research. We will continue to expand our research on this family and invite others to join us in this venture.

Conclusions

Sexual dimorphism in body size and weaponry strongly suggest a mating system involving male guarding or defense of females in the two studied *Cinetorhynchus* species. Some males are much larger than females in each species. The major weapons (pereopod 1 chelipeds) of males are significantly larger and longer than those of females. However, unlike some *Rhynchocinetes* species that have been studied, the third maxillipeds of males and females are similar in size in these two *Cinetorhynchus* species, i.e., do not appear part of male weaponry. Major chelae of males change with growth from typical female-like chelae tipped with black corneous stout setae in the smaller males to a subchelate or prehensile appendage in larger males. Puncture wounds on or regeneration of major chelipeds were observed in a number of large males. We interpret this evidence on sexual dimorphism and injuries as an indication of a temporary male mate-guarding or "neighborhoods of dominance" mating system, in which larger dominant "robustus" males fight for access and defense of reproductive females. The high overlap of male and female size and a lack of development of female breeding-dress characters clearly show that these two *Cinetorhynchus* species have separate sexes, unlike another rhynchocinetid (*R. uritai*), which is a protandric hermaphrodite. Both the present and past studies on the Rhynchocinetidae indicate extensive variation in mating systems associated with differences in population structure and male weaponry, as well as variation in sexual systems. Thus, this family may serve as a good model with which to study the evolution of mating and sexual systems in other caridean families, as well as those of other animal taxa.

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Reproductive biology of the sea anemone shrimp *Periclimenes rathbunae* (Caridea, Palaemonidae, Pontoniinae), from the Caribbean coast of Costa Rica

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Abstract

Caridean shrimps are a highly diverse group and many species form symbiotic relationships with different marine invertebrates. *Periclimenes rathbunae* is a brightly colored shrimp that lives predominantly in association with sea anemones. Information about the reproductive ecology of the species is scarce. Therefore, we collected 70 ovigerous females inhabiting the sun sea anemone *Stichodactyla helianthus* in coral reefs from the southern Caribbean coast of Costa Rica. Females produced on average 289 ± 120 embryos. The volume of recently-produced embryos was on average 0.038 mm^3, and embryo volume increased by 192% during the incubation period. The average embryo mortality during embryogenesis was 24%. The reproductive output was 0.24 ± 0.094, considerably higher than in many other pontoniine shrimps. Females carrying embryos close to hatching showed fully developed ovaries, suggesting consecutive spawning. We assume that the sheltered habitat, living on sea anemones, allows *P. rathbunae* to allocate more energy in embryo production than most other free-living caridean shrimps. This is the first record of *P. rathbunae* for Costa Rica.

Keywords

Coral reefs, fecundity, new record, reproductive output, symbioses

Introduction

Caridean shrimps are a highly diverse group within the Decapoda, comprising approximately 3438 currently valid species within 389 genera (De Grave and Fransen 2011). They inhabit a wide range of habitats (Chace 1972, Bauer 1985, Anker 2003, De Grave et al. 2008), and have different niches (Welsh 1975, Bauer 2004, Hultgren and Duffy 2012), mating behaviors (Berg and Sandifer 1984, Thiel and Hinojosa 2003, Bauer and Thiel 2011, Baeza et al. 2013), and reproductive features (Gherardi and Calloni 1993, Bauer 2000, Echeverría-Sáenz and Wehrtmann 2011, Nye et al. 2013). Caridean shrimps have been relatively well studied, mainly due to the fact that many species are valuable fishery resources (Clarke et al. 1991). Other shrimps have been targeted by the aquarium trade as ornamental species, due to their bright colors and display of associative behavior with other marine species (Calado et al. 2003a, Rhyne et al. 2009).

Many studies report on symbiotic relationships between caridean shrimps and other invertebrates, such as sponges, cnidarians, echinoderms, mollusks, crustaceans, and also with fishes (Bruce 1976, Criales and Corredor 1977, Bauer 2004). The symbiotic partner can receive cleaning services (Limbaugh et al. 1961, Criales and Corredor 1977), protection from predators (Smith 1977), burrow access (Karplus 1987), or increased nitrogen concentrations from shrimp excretions (Spotte 1996). On the other hand, caridean symbionts often benefit by protection from predators or feeding on the host tissue (Fautin et al. 1995, Silbiger and Childress 2008), and increased chances for successful reproduction (Kotb and Hartnoll 2002).

The highly diverse genus *Periclimenes* Costa, 1844 comprises approximately 152 species (De Grave and Fransen 2011). Many species of *Periclimenes* are usually associated with different marine invertebrates such as sea anemones, corals, sea stars and sea cucumbers (Bruce 2004). *Periclimenes rathbunae* Schmitt, 1924 is a brightly colored shrimp recorded from Florida (USA), Mexico, Belize, Colombia, Cuba, Turks and Caicos, Tobago, and Curaçao (Chace 1972, Román-Contreras and Martínez-Mayén 2010). The species has been found associated to a variety of different shallow-water sea anemones such as *Bartholomea annulata* (Le Sueur, 1817), *Bunodosoma granuliferum* (Le Sueur, 1817), *Condylactis gigantea* (Weinland, 1860), *Homostichanthus duerdeni* (Carlgren, 1900), *Lebrunia neglecta* Duchassaing & Michelotti, 1860 and *Stichodactyla helianthus* (Ellis, 1768) (Spotte et al. 1991, Hayes and Trimm 2008). However, it has been also collected from the gorgonian *Eunicea tourneforti* Milne Edwards & Haime, 1857 (see Criales 1980) and from dead corals (Chace 1972). Biological studies on *P. rathbunae* have focused mainly on their ecology and hosts (Spotte et al. 1991, Hayes and Trimm 2008) but information about reproductive features is scarce (Spotte 1997).

Hines (1982, 1988, 1991) studied the reproductive output (RO) of different marine decapods and reported RO values around 10% for a variety of brachyuran crab species. However, decapod species living as commensals (e.g. in bivalves and corals) and with a reduced calcification of the integument can allocate substantially more energy in embryo production (e.g. pea crabs *Zaops ostreus* (Say, 1817) and *Fabia subquadrata* Dana, 1851: Hines 1992; coral gall crab *Hapalocarcinus marsupialis* Stimpson, 1859:

Kotb and Hartnoll 2002). Here we studied fecundity and reproductive output of *P. rathbunae* in order to test the hypothesis that pontoniine shrimps living as symbionts with other invertebrates can invest more energy in embryo production that free-living shrimps but less than decapods living enclosed in other invertebrates.

Methods

Ovigerous females of *P. rathbunae* were collected during five field trips (September and October 2011, January, June and October 2012) in the Puerto Viejo-Punta Mona coral reef area (Cortés et al. 2010) within the Gandoca-Manzanillo National Wild Life Refuge, at the southern Caribbean coast of Costa Rica (Fig. 1). The sea surface temperature in all sampling months varied between 27 and 30 °C (data provided by MIO-CIMAR: http://www.miocimar.ucr.ac.cr/). The substrate was dominated by algal ridges, with low live coral cover, ranging from 8 to 16%, although this percentage has been increasing during the last decades (Cortés et al. 2010). All specimens were collected between 1-4 m depth, and were associated with the sun sea anemone, *S. helianthus* Ellis, 1768. The shrimps were collected by hand during snorkeling dives and placed individually into plastic vials. The collected specimens were stored and preserved in 70% ethanol, and subsequently transported to the laboratory at the Escuela de Biología, Universidad de Costa Rica, in San José. The shrimps were identified according to Chace (1972) and photos provided by Dr Arthur Anker. Six specimens were deposited in the Museo de Zoología of the Universidad de Costa Rica (catalog number MZUCR 3155-01). The material was collected under the sampling permit No. 181-2013 provided by SINAC-MINAET.

The total length (TL, distance between distal part of the eye socket to the distal margin of the telson excluding setae) and carapace length (CL, distance between distal part of the eye socket to the posterior margin of the carapace) were measured with the aid of Leica MS5 stereoscopic microscope equipped with a calibrated ocular micrometer. Linear regression was performed to test the relationship between TL and CL. The entire embryo mass was removed from females and photographed to count the number of embryos, using IMAGE TOOL version 3.00 developed by UTHSCSA. Here we used the term fecundity as the number of embryos carried by the female (Corey and Reid 1991). The female length and the number of embryos were correlated using linear regression analysis. Thirty embryos of each clutch were separated, and length and width of embryos were measured under a Leica CME microscope equipped with a calibrated ocular micrometer. The embryo volume was calculated using the formula for oblate spheroids $V = 1/6\ (\pi d_1 \times d_2^2)$ where d_1 is the mayor diameter, and d_2 is the perpendicular diameter (Turner and Lawrence 1979). The brood mass volume was estimated multiplying total embryo number per female by their respective average embryo volume (Echeverría-Sáenz and Wehrtmann 2011).

The stage of embryo development was assigned following the criteria described by Wehrtmann (1990): Stage I, uniform yolk, no eye pigments observed; Stage II, eye pigments start to develop; Stage III, embryo clearly visible and fully developed. The

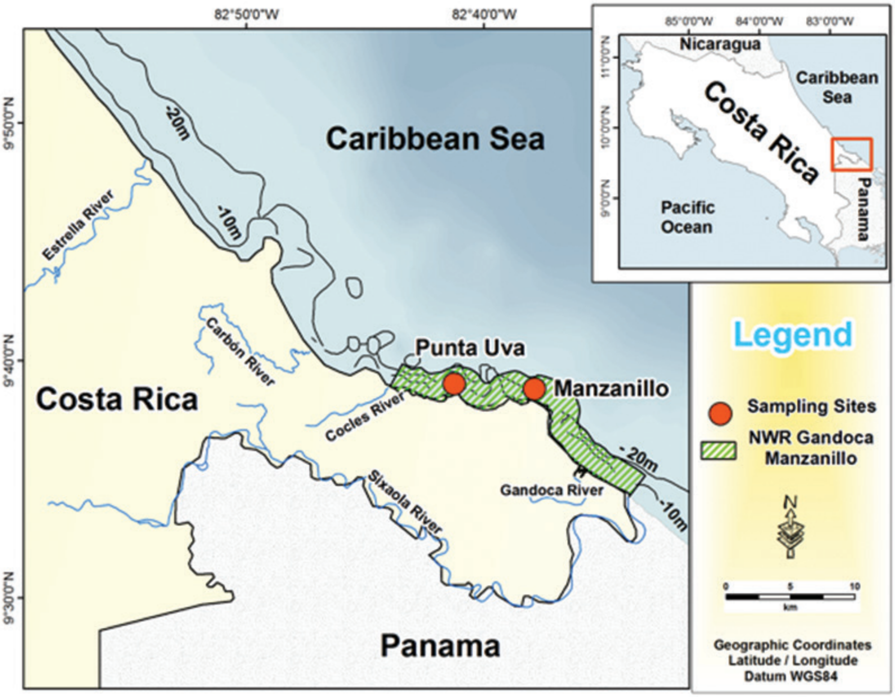

Figure 1. Sampling sites of *Periclimenes rathbunae* visited between 2011 and 2012: Punta Uva and Manzanillo beaches, Gandoca-Manzanillo National Wild-Life Refuge, Caribbean coast of Costa Rica.

number of embryos, embryo volume and brood mass volume were compared between developmental stages of the embryos using one-way analysis of variance. Females and brood masses were dried separately at 60 °C for 48 hours, and the dry weight was measured using a Sartorius TE64 analytical balance (± 0.0001) to calculate the reproductive output (RO): dry weight of total brood mass per female divided by dry weight of female without brood mass (Hines 1988, Echeverría-Sáenz and Wehrtmann 2011). The RO was estimated exclusively for females carrying recently-extruded embryos (Stage I).

The stage of ovarian development was determined following the criteria proposed by Bauer (1986): Stage 1, no noticeable development; Stage 2, vitellogenic oocytes distinct but small ovary; Stage 3, ovary filling at least half the space above the cardiac stomach; Stage 4, ovary completely filling the space above the cardiac stomach. The ovarian and embryos development were analyzed to infer the possibility of consecutive spawning (Bauer 1986, 1992).

Results

A total of 70 ovigerous females of *P. rathbunae* were analyzed; TL of these specimens was directly proportional to CL (CL = 0.1657 × TL + 0.5497; F = 313.21; DF = 69;

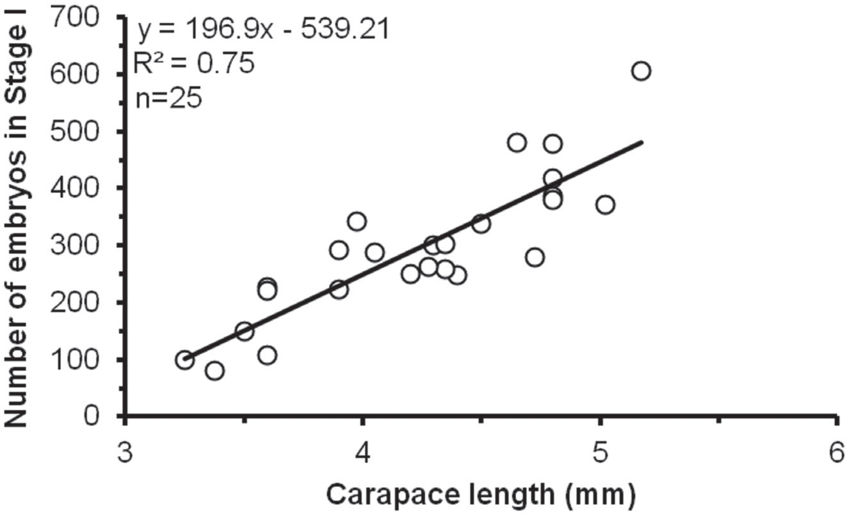

Figure 2. Relation between number of embryos in Stage I and female size of *Periclimenes rathbunae* from the Caribbean coast of Costa Rica, 2011–2012.

$P < 0.001$; $R^2 = 0.80$). Individuals ranged in size from 2.25 to 5.25 mm CL with an average of 3.98 ± 0.77 mm CL. A total of 29 females carried embryos in Stage I, 14 in Stage II, and 27 in Stage III.

Embryo number

The average number of Stage I embryos was 289 ± 120 embryos per female, with a minimum and maximum of 80 and 605, respectively. The number of recently-extruded embryos (Stage I) increased significantly with female size ($F = 69.1$; $DF = 23$; $P < 0.001$; $R^2 = 0.75$) (Fig. 2). The embryo number was significantly different between Stage I and III ($F = 3.5$; $DF = 66$; $P = 0.03$) (Table 1), but this difference was due to a significant decrease of embryo numbers from Stage II to Stage III ($F = 4.7$; $DF = 39$; $P = 0.03$). Average embryo number decreased during the incubation period by 24%.

Embryo volume and brood mass volume

Recently-produced embryos (Stage I) had an average volume of 0.038 mm^3, and those closed to hatching (Stage III) 0.073 mm^3 (Table 1), representing a volume increase of 192% during the incubation period. The average embryo volume ($F = 30.9$; $DF = 67$; $P < 0.001$) as well the average brood mass volume ($F = 4.5$; $DF = 66$; $P = 0.01$) was statistically different among the three developmental stages (Table 1). Average brood mass volume increased during embryogenesis from 10.6 to 15.1 mm^3 (Table 1), which represented a 42% increase.

Table 1. Number of analyzed specimens, mean ± standard deviation of carapace length (CL), embryo number, embryo volume, and brood mass volume according to the stage of embryo development in females of *Periclimenes rathbunae* from the Caribbean coast of Costa Rica, 2011–2012.

Stage of embryo development	n	CL (mm)	Embryo number	Embryo volume (mm^3)	Brood mass volume (mm^3)
I	29	4.2 ± 0.6	289 ± 120	0.038 ± 0.011	10.6 ± 4.6
II	14	4.3 ± 0.5	288 ± 105	0.050 ± 0.013	15.0 ± 6.8
III	27	4.2 ± 0.5	219 ± 90	0.072 ± 0.022	15.1 ± 6.7

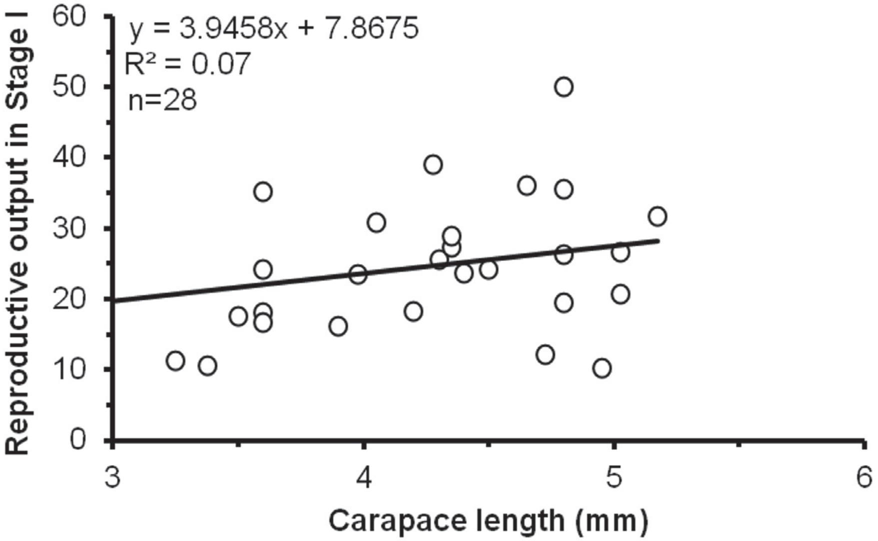

Figure 3. *Periclimenes rathbunae* from the Caribbean coast of Costa Rica, 2011–2012: relation between reproductive output (Stage I) and female size.

Reproductive output

The average RO for female *P. rathbunae* was 0.24 ± 0.094, fluctuating between 0.10 and 0.50. There was no significant correlation between RO and CL of females (F = 2.0; DF = 26; $P > 0.05$; $R^2 = 0.07$) (Fig. 3).

Reproductive activity

Ovigerous females were collected during all five field trips carried out between September 2011 and October 2012. Early ovarian stages (Stage 1–2) predominated in females carrying recently-extruded embryos (Stage I), while ovaries filled with vitellogenic oocytes (Stage 4) reached its highest occurrence in females with embryos close to hatching (Stage III) (Fig. 4).

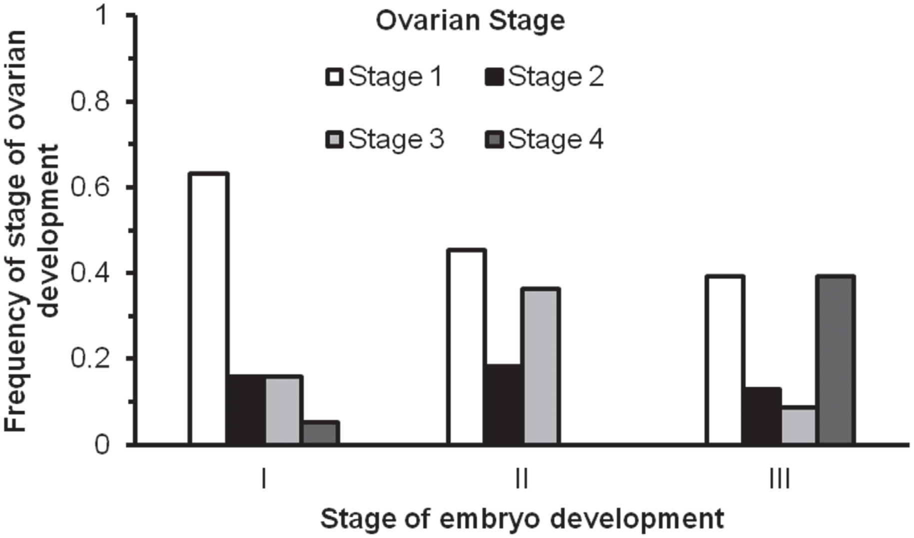

Figure 4. Frequency of stage of ovarian development in relation to the stage of embryo development from females of *Periclimenes rathbunae* from the Caribbean coast of Costa Rica, 2011–2012.

Discussion

This is the first record of *P. rathbunae* for Costa Rica. Vargas and Wehrtmann (2009) summarized the available information on marine decapod diversity in Costa Rica, but did not mention *P. rathbunae*; however, they pointed out that the Caribbean coast of Costa Rica was less studied than the Pacific coast of the country. This new record confirms the geographic distribution of the species, ranging from Florida (USA) to Curaçao (Chace 1972, Román-Contreras and Martínez-Mayén 2010).

Embryo number

The sea anemone shrimp *P. rathbunae* produces a relatively high number of offspring when compared to other pontoniine species (Table 2): only *Periclimenes ornatus* Bruce, 1969 has been reported to carry more embryos (Omori et al. 1994), but this species reaches also slightly larger sizes. Many studies on caridean shrimps revealed that fecundity is closely related to female size (for tropical palaemonids: Anger and Moreira 1998, Wehrtmann and Lardies 1999, Nazari et al. 2003, da Silva et al. 2004, Lara and Wehrtmann 2009, Meireles et al. 2013), and our data for *P. rathbunae* confirm this tendency (Fig. 2). We observed also sexual dimorphism in *P. rathbunae* with females reaching larger sizes than males (JC Azofeifa-Solano et al., unpubl data), a phenomenon well-known in many caridean shrimps (Bauer 2004), and also reported for *P. ornatus* by Omori et al. (1994) who found that males attained smaller sizes, but had larger chelae than females.

Table 2. Minimum and maximum female carapace length (CL), embryo number in Stage I, mean embryo volume and reproductive output (RO) in Stage I, habitat and study site of seven pontoniine species; NA = no data available; * = total length. ** = Value recalculated by the authors of the present study (see Discussion).

Species	CL (mm)	Embryo number	Embryo volume (mm^3)	RO (%)	Habitat	Study site	Reference
Periclimenes ornatus Bruce, 1969	3.0–6.0	10–1000	0.49 (0.06**)	NA	Sea anemone	Shikoku Island, Japan	Omori et al. (1994)
Ancylomenes pedersoni (Chace, 1958) Reported as *Periclimenes anthophilus*	NA	33–80	0.05	NA	Sea anemone	Bermuda	Spotte (1999)
Ancylomenes pedersoni (Chace, 1958) Reported as *Periclimenes pedersoni*	NA	78–221	0.11	NA	Sea anemone	Bahamas	Spotte (1999)
Periclimenes pandionis Holthuis, 1951	2.84–4.0	67–259	0.05	NA	Coral reef	Indian River, Florida	Corey and Reid (1991)
Periclimenes patae Heard & Spotte, 1991	3.3–4.2	10–35	NA	NA	Gorgonian	Turks and Caicos and Florida Keys	Heard and Spotte (1991)
Periclimenes yucatanicus (Ives, 1891)	3.52–5.73	12–333	NA	NA	Sea anemone	West Indies and south Florida	Spotte (1997)
Phycomenes siankaanensis (Martinez-Mayén & Román-Contreras, 2006)	1.91–3.2	23–141	0.056	NA	Sea grass meadow	Quintana Roo, Yucatan Peninsula, Mexico	Martínez-Mayén and Román-Contreras (2009)
Periclimenes rathbunae Schmitt, 1924	2.25–5.25 (12.3–22.6*)	80–605	0.038	24.0 ± 0.09	Sea anemone	Limón, Costa Rica	Present study

Embryo volume and brood mass volume

The average embryo volume of *P. rathbunae* is in the range of most values reported for other pontoniine species (Table 2). However, *P. ornatus* in southwest Japan produces considerably larger embryos with a maximum volume of 0.49 mm^3 (Omori et al. 1994). However, these authors did not explain how they calculated the embryo volume. Using the average values for embryo length and width provided by Omori et al. (1994), and applying the same equation as used in the present study (Turner and Lawrence 1979), average embryo volume of *P. ornatus* would be 0.06 mm^3, which is in the range reported for other pontoniine species, including *P. rathbunae* (Table 2). Both species, *P. rathbunae* and *P. ornatus*, share similar size ranges and live associated with sea anemones in tropical and subtropical waters (Chace 1972, Omori et al. 1994). Most species of pontoniine shrimps producing these relatively small embryos, including *P. rathbunae*, probably have an extended larval development. There is still a great lack of knowledge concerning the complete larval development of most of the pontoniine species; however, dos Santos et al. (2004) cultivated *Periclimenes sagittifer*

Norman, 1861 in the laboratory and described eight zoeal stages, corroborating an extended larval period.

Periclimenes rathbunae lost during the incubation period on average 24% of the initially-produced embryos, while embryo volume increased by 192%. Brood loss in *P. rathbunae* was similar to that reported for other palaemonid freshwater shrimps: approximately 23% in *Palaemon pandaliformis* (Stimpson, 1871) and *Macrobrachium acanthurus* (Wiegmann, 1836) (Kuris 1991, Anger and Moreira 1998) and was slightly higher than the 17.2% of brood loss observed in the marine palaemonid *Palaemon gravieri* (Yu, 1930) (see Kim and Hong 2004). Furthermore, it was higher than the brood loss reported for the sponge-dwelling snapping shrimp *Synalpheus yano* (Ríos & Duffy, 2007) from Panama (Hernáez et al. 2010). The volume increase of caridean shrimp embryos during the incubation period ranges from 3.9 to 155.9% (Corey and Reid 1991); thus the observed value for *P. rathbunae* is considerably higher than this range. The combination of relatively low brood loss and substantial embryo volume increase during embryogenesis in *P. rathbunae* suggests that the physical space available for embryo attachment is sufficient to accommodate and maintain the clutch until hatching. The association with the sea anemone and efficient parental care during the incubation period may provide favorable conditions for the embryo development, and thus reducing brood loss during the embryogenesis. Such an interpretation is in agreement with similar observations in *S. yano*, a pair-living and sponge-dwelling alpheid species from tropical waters (Hernáez et al. 2010).

Reproductive output

The RO is a widely used instrument to document and compare inter- and intra-specifically energy allocation in offspring production of decapod crustaceans (Clarke et al. 1991, Hines 1991, Lardies and Wehrtmann 1996, Anger and Moreira 1998, Terossi et al. 2010, Echeverría-Sáenz and Wehrtmann 2011). To our best knowledge, here we report the first RO value for any pontoniine species (Table 2). The RO of *P. rathbunae* females (0.24) was higher than that reported for some free-living marine shrimps: 14.4 ± 2.5% for *Palaemon northropi* (Rankin, 1898) (see Anger and Moreira 1998) and 17.8 ± 6.0% for *Heterocarpus vicarius* Faxon, 1893 (see Echeverría-Sáenz and Wehrtmann 2011); and freshwater shrimps: 18.6 ± 3.0% for *P. pandaliformis* (Stimpson, 1871) (see Anger and Moreira 1998), 19.1 ± 4.5% for *M. acanthurus* (Wiegmann, 1836) (see Anger and Moreira 1998), 21.7 ± 6.6% for *Macrobrachium olfersii* (Wiegmann, 1836) (see Anger and Moreira 1998), and 3.6 ± 1.9% for *Atya scabra* (Leach, 1816) (see Herrera-Correal et al. 2013). These results suggest that marine caridean shrimps species living in association with other invertebrates are able to invest on average more energy in brood production than free-living species. Nevertheless, we suggest analyzing the RO within the subfamily Pontoniinae between free living and symbiont species in order to test if a sheltered habitat is related to an increase in RO in this diverse group of shrimps.

The reduction of the calcification of the exoskeleton results in a minimized dry weight of the decapod species, thus increasing its relative brood weight (Hines 1992; Kotb and Hartnoll 2002). The symbiont *H. marsupialis*, a species which provokes gall development in its host coral has a reproductive investment per brood of 59% (Kotb and Hartnoll 2002), and this value is still lower than those published for symbiotic decapods, such as the pea crabs *Z. ostreus* (66%) and *F. subquadrata* (97%) (Hines 1992). While the coral gall crab and the pea crabs live protected within galls of corals and bivalves, respectively, species like *P. rathbunae* are associates on other invertebrates, more exposed to predation and wave action than the above-mentioned crab species. This may explain the relatively high RO value in *P. rathbunae* compared to free-living decapods, but substantially lower RO-value when compared to symbiotic decapods living enclosed within their host. Additional studies with decapod species living in association with other invertebrates are needed to substantiate the hypothesis of increasing energy allocation in brood production with increasing degree of protection provided by the host.

Reproductive activity

Relatively elevated and stable temperatures in tropical seas may allow year-round reproduction of marine decapods (Bauer 1986, 1992). Our results concerning the relation between the state of ovarian development and stage of embryo development as well as the fact that ovigerous females of *P. rathbunae* were encountered throughout the sampling period confirms that this species has continuous reproduction (Mossolin and Bueno 2002). Bauer (1992) studied reproductive patterns in different caridean shrimps, including *Cuapetes americanus* (Kingsley, 1878) associated with sea grass meadows in Puerto Rico; his results revealed that all of these caridean species go through continuous cycles of ovarian maturity and spawning until they die. Our results corroborate the generalized pattern of continuous reproduction in tropical shallow-water shrimps and confirm *P. rathbunae* as an iteroparous species.

In recent years, the pressure on ornamental species has increased, and this includes also decapod species, which are highly popular among aquarium hobbyists (Calado et al. 2003a). As far as we know, *P. rathbunae* has not been harvested yet at the Caribbean coast of Costa Rica; however, its eye-catching color pattern and the fact that the shrimp lives in association with sea anemones makes it a potential candidate for aquarium hobbyists, just as numerous other pontoniine species (see Calado et al. 2003a). In order to minimize possible negative impacts caused by the harvest of wild marine species such as *P. rathbunae* (see Wood 2001), additional information on larval development is needed to cultivate the early life stages under controlled laboratory conditions (Calado et al. 2003b). Moreover, many other aspects of the ecology of *P. rathbunae* remain to be studied, e.g., mating behavior, recruitment, settlement on the host species as well as other details about the association of the shrimp with its host.

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Embryo production in the sponge-dwelling snapping shrimp *Synalpheus apioceros* (Decapoda, Alpheidae) from Bocas del Toro, Panama

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Abstract

Caridean shrimps of the genus *Synalpheus* are abundant and widely distributed in tropical and subtropical regions, but knowledge of their reproductive biology remains scarce. We report reproductive traits of *Synalpheus apioceros* from Bocas del Toro, Panama, based on collections in August 2011. The 46 ovigerous females that were analyzed ranged in size from 3.8 to 7.4 mm in carapace length. Fecundity varied between 8 and 310 embryos and increased with female size. Females invested 18.6 ± 10.3% of their body weight in Embryo production. Embryo volume increased considerably (77.2%) during embryogenesis, likely representing water uptake near the end of incubation period. Compared to *Synalpheus* species with abbreviated or direct development, *S. apioceros* produced substantially smaller embryos; however, *S. apioceros* seems to have a prolonged larval phase with at least five zoeal stages, which may explain the combination of relatively small and numerous embryos. We did not find nonviable, minute, chalky embryos, previously reported for *S. apioceros* specimens obtained from the northwestern Gulf of Mexico, which supports the hypothesis that the production of this type of embryos may be a physiological response of this warm-water species to the temperature decrease near to its latitudinal range limit.

Resumen

Aunque los camarones carídeos del genero *Synalpheus* son muy abundantes y se encuentran ampliamente distribuidos en regiones tropicales y subtropicales, el conocimiento sobre su biología reproductiva es escaso. En este estudio reportamos algunas características reproductivas de especímenes de *Synalpheus apioceros* de Bocas del Toro, Panamá, colectados en Agosto del 2011. El largo del caparazón de las 46 hembras ovígeras analizadas se encuentra en un rango de 3.8 y 7.4 mm. La fecundidad varió entre 8 y 310 huevos, aumentando con el tamaño de la hembra. Las hembras invirtieron 18.6 ± 10.3% de su peso corporal en la producción de huevos. El tamaño de los huevos aumentó considerablemente (77.2%) durante la embriogénesis, probablemente por la absorción de agua al final del periodo de incubación. Comparado con especies de *Synalpheus* que presentan desarrollo abreviado o directo, *S. apioceros* produce huevos considerablemente más pequeños; sin embargo *S. apioceros* parece tener una fase larval prolongada, con al menos cinco estadios larvales, lo que podría explicar que los huevos sean relativamente pequeños y numerosos. No encontramos los huevos anómalos, no viables, que previamente se habían reportado para especímenes obtenidos en el Golfo de México, lo cual apoya la hipótesis de que la producción de este tipo de huevos puede ser una respuesta fisiológica a la disminución de temperatura cerca del límite latitudinal de esta especie habitante de aguas cálidas.

Keywords

Central America, embryo volume, fecundity, incubation period, reproductive output, water uptake

Introduction

Reproductive traits of crustacean species offer relevant information about their life history strategies (Sastry 1983, Ramirez-Llodra 2002). Fecundity, defined as the number of offspring produced by a female in a determined time period, is directly related to energy allocation and is essential in estimating reproductive potential of a population (Ramirez-Llodra 2002, Zare et al. 2011). Embryo size is an indicator of the energy allocation, duration of embryogenesis and type of larval development (Dardeau 1984, Ramirez-Llodra 2002). The reproductive output quantifies the energetic investment of a species in embryo production (Hines 1991, Anger and Moreira 1998).

Caridean shrimps of the genus *Synalpheus* are distributed worldwide with estimates of about 150 valid species (Dardeau 1984, Ríos and Duffy 2007, Macdonald et al. 2009, Hermoso-Salazar and Solis-Weiss 2010, Hultgren et al. 2010, 2011, De Grave and Fransen 2011, Anker et al. 2012). Whether free-living or (more typically) living as facultative or obligate symbionts, these pistol or snapping shrimps commonly inhabit sponge cavities, coral reefs, rocks, grass beds, or tide pools, and may live associated with hard or soft corals, ascidians, bryozoans, and crinoids (Corey and Reid 1991, Ríos and Duffy 2007, Macdonald et al. 2009, Anker et al. 2012).

Most studies on this genus have focused on geographical distribution (Hermoso-Salazar and Hendrickx 2005a, Macdonald et al. 2009, Bacci et al. 2010, Hultgren et al. 2011), taxonomy and species revision (Duffy 1996a, Ríos and Duffy 1999, Hermoso-Salazar et al. 2005, Hermoso-Salazar and Hendrickx 2005a,b, Macdonald and Duffy

2006, Anker and Toth 2008, Anker et al. 2012), phylogenetics (Morrison et al. 2004, Hultgren and Duffy 2011), or behavior and eusociality (Duffy 1996b,1998, Duffy and Macdonald 1999, Duffy et al. 2000, 2002). Despite the large amount of information accumulated about these interesting snapping shrimps, little is known about their reproductive ecology. Corey and Reid (1991) provided some data on the fecundity of six species of *Synalpheus*, and Hernáez et al. (2010) described reproductive features and the effect of bopyrid parasitism on embryo production in *S. yano* Rios & Duffy. Dobkin (1969) studied the larval development of *Synalpheus apioceros* Coutière and concluded that this species has a prolonged larval phase. The only published study regarding embryo production of *S. apioceros* mentioned the presence of anomalous small embryos and described the pattern of seasonality of ovigerous females from the northwestern Gulf of Mexico but did not address fecundity (Felder 1982).

Synalpheus apioceros is widely distributed in the western Atlantic (Gulf of Mexico; Florida; Bahamas), throughout the Caribbean Sea (e.g., Panama, Puerto Rico, Mexico, Venezuela etc.), Suriname, and Brazil (Amapá to Santa Catarina). Assuming that we are dealing with a single species, it can be found in association with different hosts (see Anker et al. 2012 for revision). The species thus represents an excellent candidate to study reproductive adaptations of those *Synalpheus* spp. that typically live in heterosexual pairs but with a variety of hosts. The present study addresses reproduction in *S. apioceros* by describing fecundity, reproductive output, volume and water content of the embryos at different embryonic stages for a tropical Caribbean population.

Methods

Ovigerous females of *Synalpheus apioceros* were collected by hand (August 2011) from an area near the Smithsonian Tropical Research Institute (STRI) marine station (09°20'N, 82°14'W), at Bocas del Toro, on the Caribbean coast of Panama. Shrimps were found in the red-orange sponge *Lissodendoryx colombiensis* Zea & van Soest, growing on jetty pilings and mangrove roots. In the laboratory, ovigerous females were extracted from the sponge canals and stored individually to avoid mixing and loss of the embryos and then preserved in ethanol (70%). Voucher specimens were deposited in the Crustacean Collection of the Museo de Zoología - Universidad de Costa Rica (MZUCR) under catalog number MZUCR 3128-01.

Carapace length (CL) of ovigerous females was measured (± 0.1 mm) under a stereomicroscope with an ocular micrometer, from the tip of the rostrum to the posterior margin of the carapace. The entire embryo mass from each female was detached from the pleopods and embryos were classified into three stages (I–III) according to the shape and development of the abdomen and eyes (Wehrtmann 1990): Stage I: almost round embryo, uniform yolk, no visible eye pigments; Stage II: ovoid embryo, eye pigments barely visible; Stage III: ovoid embryo, eye fully developed, abdomen free.

Ten embryos of each female were randomly selected to measure the length (*a*) and width (*b*) under a stereomicroscope equipped with an ocular micrometer; these data were used to determined the embryo volume (EV) with the formula $EV = 1/6 \times a \times b^2 \times \pi$ (Turner and Laurence 1979). The wet weight (WW) of the embryo mass was determined with an analytical balance and the embryos then oven-dried at 60 °C for 24 hours to obtain embryo mass dry weight (EDW). Embryo water content was calculated by subtracting EDW from WW.

Due to possible embryo loss during the incubation period (Terossi et al. 2010), only females carrying recently-produced embryos (Stage I) were used to estimate fecundity and reproductive output (RO). Fecundity was considered as the total number of embryos present on each individual. The dry weight of the females (FDW) carrying Stage I embryos was obtained to calculated the RO applying de formula proposed by Clarke et al. (1991): $RO = EDW_{(\text{Stage I})}/FDW$.

Data were analyzed with the statistical software SPSS v.20.0. The assumption of normality on the size distribution of the individuals was tested using the Kolmogorov-Smirnov test. Linear regressions and Pearson's correlation analyses were applied to determine the relation between CL and fecundity, and CL and RO. One-way Analyses of Variance (ANOVA) were used to compare embryo volume and water content among the three stages of development.

Results

A total of 46 ovigerous females were analyzed; the majority of them (21 total or 45.7%) carried embryos at Stage I, 10 (21.7%) in Stage II and 15 females (32.6%) in Stage III. The size frequency distribution was normal (Kolmogorov-Smirnov test, $KS = 0.11$, $p > 0.05$). The mean CL of the individuals was 5.4 ± 0.8 mm, ranging from 3.8 to 7.4 mm, and 43.5% of the ovigerous females were in the intermediate size class of 5.0–5.9 mm (Fig. 1).

Fecundity in Stage I ranged from 8 to 310, and increased with female size (Pearson's correlation, $r = 0.68$, $p < 0.05$) (Fig. 2). Females within the same size-class presented different number of embryos (Fig. 2 and Table 1). Energy invested in embryo production by the females was not related to female size (Pearson's correlation, $r = 0.26$, $p > 0.05$). The embryo mass comprised on average 18.6 ± 10.3% (4.8–40.1%) of the dry body weight of the females.

Embryos were slightly oval with mean diameters ranging from 0.63 ± 0.04 mm (Stage I) to 0.77 ± 0.06 mm (Stage III). Embryo volume differed significantly (ANOVA, $F = 369.25$, $p < 0.05$) between the stages of development, with an overall volume increase during the incubation period of 77.2% (Table 2). During the embryogenesis, the water content increased substantially (ANOVA, $F = 82.60$, $p < 0.05$) from Stage I (59.0 ± 5.9%) to Stage III (82.9 ± 3.6%). Dry mass remained almost constant throughout the incubation period (ANOVA, $F = 0.10$, $p > 0.05$) (Table 2).

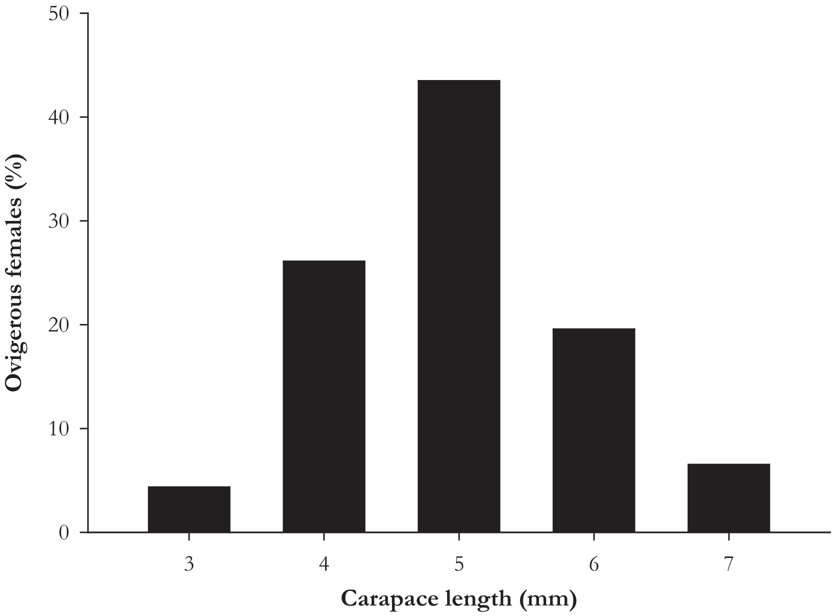

Figure 1. Frequency distribution of carapace length of ovigerous females of *Synalpheus apioceros* (N = 46), Bocas del Toro, Panama.

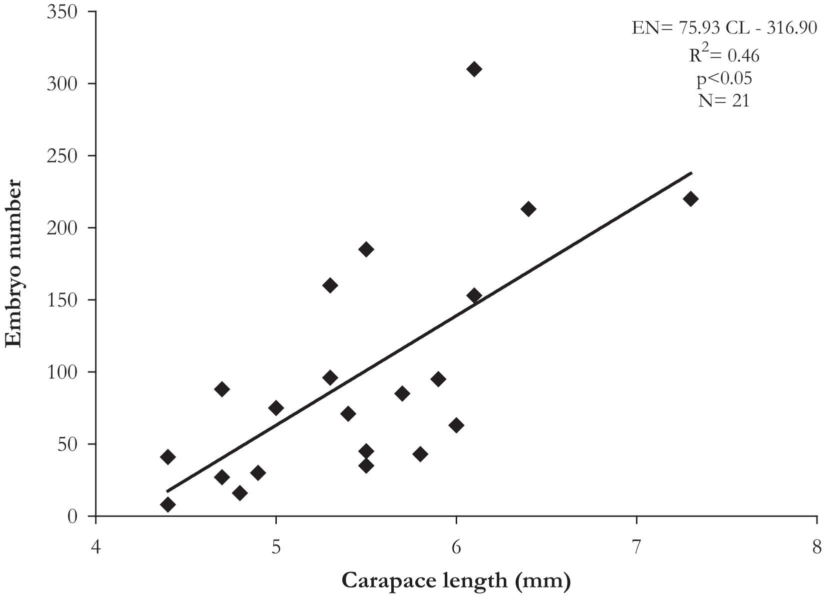

Figure 2. Relation between carapace length and fecundity of *Synalpheus apioceros* females carrying stage I embryos, Bocas del Toro, Panama.

Table 1. Fecundity by size class in females carrying recently-produced embryos (Stage I) for *Synalpheus apioceros*, Bocas del Toro, Panama.

Size class (mm)	Mean embryos number
4.0–4.9 (N = 6)	34.8 ± 28.5
5.0–5.9 (N =10)	88.6 ± 49.6
6.0–6.9 (N = 4)	184.8 ± 103.8
7.0–7.9 (N = 1)	220

Table 2. Embryo volume, weight, and water content of different embryonic development stages of *Synalpheus apioceros*, Bocas del Toro, Panama.

Embryo features	Stage I (N = 20)	Stage II (N = 8)	Stage III (N = 9)
Embryo volume (mm^3)	0.101 ± 0.015	0.136 ± 0.018	0.179 ± 0.034
Wet weight (µg)	74.0 ± 12.5	128.2 ±10.5	173.9 ± 19.8
Dry weight (µg)	30.1 ± 5.3	29.8 ± 1.5	29.3 ± 5.2
Water content (µg)	43.9 ± 10.0	98.4 ± 9.4	144.6± 21.0
% Water content	59.0 ± 5.9	76.6 ± 2.0	82.9 ± 3.6

Discussion

The size of ovigerous females of *Synalpheus apioceros* from Bocas del Toro is within the range for congeneric species (Table 3). Fecundity in *S. apioceros* increased with female size, which is in agreement with similar observations from other caridean shrimp (Corey and Reid 1991, Anger and Moreira 1998, Lara and Wehrtmann 2009). Female size is postulated to determine the number of embryos produced by the individual, as larger females have more space available for the attachment of embryos on their pleopods (Lara and Wehrtmann 2009).

Fecundity variation within the same size class is a well-known phenomenon in decapods, including alpheid shrimps (Lardies and Wehrtmann 1997, 2001, Pavanelli et al. 2008). Such variability as observed in *S. apioceros* could be due to multiple spawnings during a single reproductive season (Miranda et al. 2006, Mantelatto et al. 2007). Also, the presence of primiparous females, which tend to produce fewer embryos than multiparous ones (Somerton and Meyers 1983, Mantelatto et al. 2007) could explain the variability of embryo number within the same size class.

The energy invested in embryo production, estimated by reproductive output (RO), was not related to their size in *S. apioceros*. Average RO in alpheid shrimp is highly variable, ranging from 7 to 35% (Lardies and Wehrtmann 1997, 2001, Pavanelli et al. 2008, 2010, Hernáez et al. 2010). Although the RO of *S. apioceros* is lower (18.6%) than that reported for another tropical sponge-dweller, *S. yano* (35%: Hernáez et al. 2010), it is higher than that observed in other subtropical alpheid species (Pavanelli et al. 2008, 2010). The elevated RO values in sponge-dwelling alpheids may represent an adaptation to their life style: the relatively protected habitat may favor energy investment in embryo production, which also favors enhanced larval pro-

Table 3. Mean carapace length, embryo number, and embryo volume for ten species of sponge-dwelling *Synalpheus*.

Species	N	Carapace length (mm)	Embryo number	Embryo volume (mm^3)	Reference
Synalpheus agelas	5	5.0 (4.2–5.6)	42.4 (16–65)	0.23	Corey and Reid (1991)
S. brooksi	10	3.9 (3.4–4.5)	5.8 (3–11)	0.50	
S. fritzmuelleri	13	4.9 (3.8–6.5)	173.4 (39–484)	0.09	
S. herricki	4	4.5 (3.5–5.12)	45.8 (11–81)	0.22	
S. longicarpus	21	6.9 (5.5–8.0)	195.4 (27–349)	0.17	
S. pectiniger	31	4.2 (3.5–4. 6)	9.9 (4–17)	0.75	
S. chacei	2	3.7	16	0.15	Hernáez et al. (2010)
S. idios	4	3.9 ± 0.2 (3.7–4.2)	12.0 ± 4.3 (9–18)	1.04 ± 0.23 (0.70–1.20)	
S. yano	84	5.6 ± 1.2 (3.7–9.6)	98.0 ± 64.6 (6–246)	0.15 ± 0.08 (0.06–0.51)	
S. apioceros	46	5.4 ± 0.8 (3.8–7.4)	97.8 ± 80.1 (8–310) N = 21 (Stage I)	0.13 ± 0.04 (0.07–0.24) N = 37	Present study

duction and thus probability of recolonization in niches restricted to certain hosts. This interpretation is supported by studies on pinnotherid crabs living in different hosts, which showed extremely high RO values (*Pinnotheres ostreum* Say: 66%; *Fabia subquadrata* Dana: 97%; Hines 1992). Thus, it might be postulated that decapod species living associated with other organisms generally have a higher RO than free-living species, a pattern warranting additional studies.

The embryo volume in *S. apioceros* is within the range reported for other alpheid shrimp (Corey and Reid 1991, Lardies and Wehrtmann 1997, Pavanelli et al. 2008, 2010, Hernáez et al. 2010). Several other species of *Synalpheus* produce substantially larger embryos than does *S. apioceros* (see Corey and Reid 1991), but these species have an abbreviated or direct development. In contrast, *S. apioceros* seems to have a prolonged larval phase with at least five zoeal stages (Dobkin 1969), which may explain the presence of relatively small and numerous embryos.

Felder (1982) studied reproductive features of *S. apioceros* in the northwestern Gulf of Mexico (26°49.0'N, 97°19.3'W), close to the northern limit of the geographical distribution range of the species. He reported the presence of nonviable, minute, chalky embryos, and suggested that the production of this type of embryos may be a physiological response of this warm-water species to the temperature decrease near to its latitudinal range limit. This hypothesis is perhaps supported by the present data, because we did not detect in any of the females examined herein this type of minute embryos. We propose that laboratory experiments with *S. apioceros* be conducted to determine if lowered maintenance temperatures can provoke the production of these nonviable, minute embryos and if higher temperatures can alternatively eliminate their occurrence. Should ongoing elevation of temperatures in northern extremes of range for these tropical decapods reduce production of non-viable embryos, and thus enhance effective fecundity, this could reveal an underappreciated dynamic of coastal ocean warming.

Embryo volume of *S. apioceros* increased considerably (77.2%) during the incubation period. This is a common phenomenon in decapod species and is probably related to water uptake over the course of embryogenesis (Lardies and Wehrtmann 1997, Petersen and Anger 1997, Wehrtmann and Lardies 1999, Lara and Wehrtmann 2009), as also observed incrementally in *S. apioceros* (Table 2). At the end of the incubation period, the embryo starts to swell due to osmotic changes (Figueiredo et al. 2008), while the embryo membrane shows a decrease in thickness with a concomitant increase in elasticity, thus favoring the hatching process of the embryo (Davis 1981).

While we here provide novel information on reproduction in the sponge-dwelling alpheid shrimp *S. apioceros*, it is based on a limited sampling period and a single locality. We thus regard our work to date as a starting point from which we and others might build comparative studies. Conspecific populations can be readily sampled across latitudes and temperature regimes, as well as over varied seasons, applying the methods we have used and enabling comparative analyses. Such work can both reveal life history strategies that have evolved in these host-dependent shrimp species and shed light on what ranges of reproductive variability might be expected due to environmental interactions in this era of global coastal ocean change.

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Reproductive traits of the symbiotic pea crab *Austinotheres angelicus* (Crustacea, Pinnotheridae) living in *Saccostrea palmula* (Bivalvia, Ostreidae), Pacific coast of Costa Rica

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Abstract

Pea crabs of the family Pinnotheridae exhibit a symbiotic life style and live associated with a variety of different marine organisms, especially bivalves. Despite the fact that pea crabs can cause serious problems in bivalve aquaculture, the available information about the ecology of these crabs from Central America is extremely limited. Therefore, the present study aimed to describe different reproductive features of the pinnotherid crab *Austinotheres angelicus* associated with the oyster *Saccostrea palmula* in the Golfo de Nicoya, Pacific coast of Costa Rica. Monthly sampling was conducted from April to December 2012. Average carapace width (CW) of the 47 analyzed ovigerous females was 7.62 mm. The species produced on average 2677 ± 1754 recently -extruded embryos with an average volume of 0.020 ± 0.003 mm^3; embryo volume increased during embryogenesis by 21%, but did not vary significantly between developmental stages. Brood mass volume varied greatly (between 11.7 and 236.7 mm^3), and increased significantly with female CW. Females invested on average 76.7% (minimum: 21.7%; maximum: 162.8%) of their body weight in brood production, which confirms a substantially higher energy allocation for embryo production in pinnotherid crabs compared to free-living decapods.

Keywords

Central America, fecundity, reproductive output, symbiosis

Introduction

Symbiotic relationships in marine organisms are a well-documented phenomenon (Roughgarden 1975, Dos Santos-Alves and Pezzuto 1998, Boltaña and Thiel 2001, Thiel and Baeza 2001, Sotka 2005, Baeza 2007, Glynn 2013). The evolution of these symbiotic associations is shaped by cost and benefit aspects for both partners (Roughgarden 1975, Baeza 2007), but the role of ecological features responsible for the evolution of this lifestyle remains unclear (Baeza 2007). According to different studies (Baeza 2007, Ory et al. 2013), predation pressure is one of the principal forces driving the evolution of symbiotic associations in decapods. Small marine decapods commonly live associated with anemones, echinoderms and a variety of other invertebrates, which may serve as refuge from predation, food source or mating site (Criales 1984, Bauer 2004, Wirtz et al. 2009, Ory et al. 2013). However, studies on symbiotic decapods are still scarce, especially in those cases where environmental conditions hamper direct observations (Baeza 1999).

The Pinnotheridae is a highly diverse family, currently with 52 genera and more than 300 described species (Ng et al. 2008, De Grave et al. 2009, Palacios-Thiel et al. 2009). Members of this family are known to exhibit symbiotic relationships with numerous other invertebrates, including mollusks, polychaetes, echinoderms, brachiopods, and other decapods (Manning and Morton 1987, Feldmann et al. 1996, Baeza 1999, Lardies and Castilla 2001, McDermott 2006, Peiró and Mantelatto 2011, Trottier et al. 2012). The type of symbiotic relationships ranges from parasitism to commensalism, and pinnotherids live in a facultative and/or obligate association with their hosts (Silas and Alagarswani 1965, Schmitt et al. 1973, Stevens 1990, Hamel et al. 1999). Detailed information about many aspects of the association between pinnotherid crabs and their host remain to be studied (McDermott 2006, Peiró et al. 2011). Several studies addressed different reproductive aspects of these symbiotic crabs, including the morphology of the reproductive system (Becker et al. 2011, 2012, 2013), mating system (Dos Santos-Alves and Pezzuto 1998, Baeza 1999, Peiró et al. 2013), intraspecific latitudinal effects on different reproductive attributes (Lardies and Castilla 2001), as well as fecundity (Báez and Martínez 1976, Hines 1992, Cabrera Peña et al. 2001, Lardies and Castilla 2001). Our knowledge about energy allocation in embryo production of pinnotherid crabs is scarce; however, results obtained from several species of pea crabs clearly demonstrated that brood masses of these crabs are extraordinarily large in relation to female body size when compared to free-living decapods (Hines 1992, Lardies and Castilla 2001). This high investment in brood production is related to two unique features of the pea crabs (Hines 1992): (1) their ovaries cover not only the cephalothorax, but extend into the abdomen; (2) the exoskeleton of females is not well calcified, which allows that the body is distensible during yolk accumulation.

The pinnotherid crab *Austinotheres angelicus* Lockington, 1877 (Fig. 1) is distributed along the Pacific coast from the Gulf of California, Mexico, to Colombia, and lives in association with oysters (Campos 2002). Life history aspects of this species are virtually unknown, and therefore, the present study aimed to describe fecundity, embryo

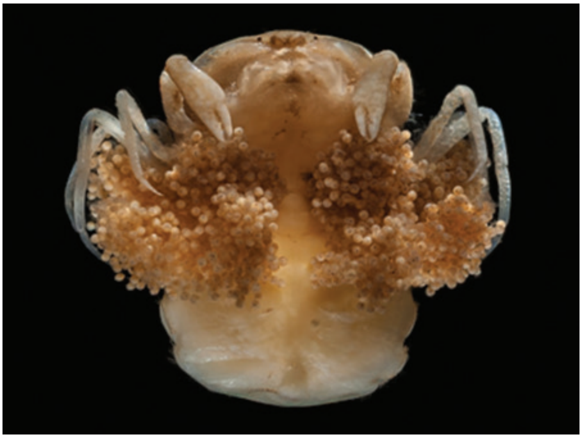

Figure 1. Ovigerous female of *Austinotheres angelicus* from the Golfo de Nicoya, Pacific coast of Costa Rica.

volume, and reproductive output of *A. angelicus* living in the oyster *Saccostrea palmula* (Carpenter, 1857) in the Golfo de Nicoya, Pacific coast of Costa Rica. The results of this study will broaden our knowledge of the evolution of reproductive strategies in decapods adapted to live in association with other marine invertebrates.

Methods

Field work

The study site was Punta Morales, Golfo de Nicoya (Fig. 2), located at the Pacific coast of Costa Rica. The location is a sandy beach with a rocky intertidal zone, surrounded by mud flats and mangrove swamps (Rojas and Vargas 2008). Monthly sampling was carried out between April and December 2012; collections were conducted during diurnal low tides. During each sampling, 30 oysters (*S. palmula*) were collected, preserved in ethanol (70%), and transported to the laboratory of the Unidad de Investigación Pesquera y Acuicultura (UNIP) of the Centro de Investigación en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica, in San José. Voucher specimens of *A. angelicus* were deposited in the collections of the Museo de Zoología, Universidad de Costa Rica (MZUCR 3281-01, 3282-01, 3283-01, 3284-01, 3285-01, 3286-01, 3287-01, 3288-01, 3289-01).

Laboratory analyses

Morphometric measurements of oysters (height, length, and thickness) were obtained with a digital caliper (± 0.01 mm); the three morphometric variables were multiplied in order to calculate the approximate volume of each oyster (OV). Each individual of

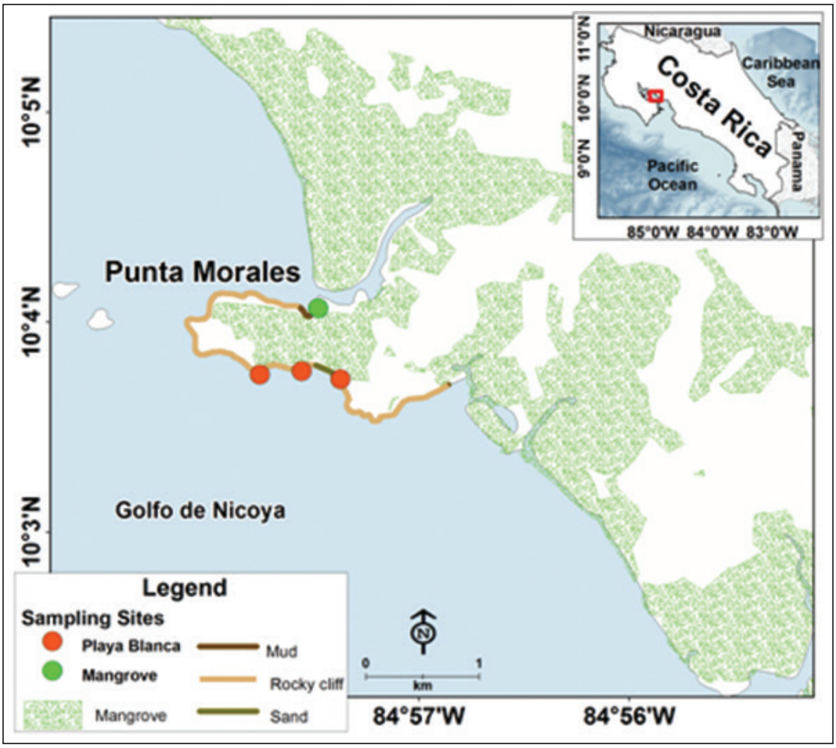

Figure 2. Sampling sites in Punta Morales, Golfo de Nicoya, Pacific coast of Costa Rica.

S. palmula was carefully opened and inspected for associated pea crabs. The carapace length (CL: distance between distal part of the eye socket to the posterior margin of the carapace), carapace width (CW: distance between lateral margins of the carapace), abdomen length (AL: distance between posterior margin of the carapace to the distal part of the abdomen) and abdomen width (AW: distance between lateral margins of the abdomen) were measured with the aid of a Leica MS5 stereoscopic microscope equipped with a calibrated ocular micrometer.

The entire brood mass was detached from ovigerous females (n = 47), evenly distributed on a Petri dish, and photographed (Benq GH650). These images were analyzed subsequently with the program ImageJ® versión 1.46r to count the total number of embryos carried by each ovigerous female. Embryos were staged according to the following criteria (Wehrtmann 1990): Stage I, uniform yolk, no eye pigments observed; Stage II, eye pigments scarcely visible; Stage III, embryo with eye pigments clearly visible and fully developed. Embryo volume (EV) was estimated using the equation (1) proposed by Corey and Reid (1991):

$$EV = \left(\frac{b}{2}\right)^2 \times a \times \pi \qquad (1)$$

where *a* is the major diameter, and *b* is the perpendicular diameter. A total of 15 embryos were measured from each female, and the average embryo volume was multiplied by the total number of embryos per female to calculate brood mass volume.

Females and their separated brood masses were dried at 60 °C for 48 hours, and dry weights were measured to calculate the reproductive output (RO): dry weight of total brood mass per female / dry weight of females without brood mass (Hines 1988). The RO was estimated exclusively for females carrying recently extruded embryos (Stage I).

Data analyses

The Kruskal-Wallis test was applied to detect possible differences in both brood numbers between the three embryonic stages, and embryo volume during embryogenesis. Simple linear regressions and the Pearson correlation coefficient were calculated to determine the relation between both host morphometry (OV: volume of the oyster) and CW of *A. angelicus* (considering exclusively females with recently -produced embryos), fecundity (exclusively embryos in Stage I) and the following morphometric variables of the female: CW, CL, AW, and AL; and finally brood mass volume and CW of ovigerous females. All statistical analyses were carried out with JMP® version 7.0.

Results

The CW of the ovigerous female crabs averaged 7.62 mm, and ranged in size from 5.02 to 14.25 mm (Table 1). Mean fecundity was 2677 in Stage I and 4890 embryos in females with brood masses in Stage III (Table 1); however, mean embryo number did not vary significantly between developmental stages ($\chi^2 = 2.57$; gl = 2; $p = 0.28$). The CW of *A. angelicus* was significantly influenced by the volume of its host, *S. palmula* ($t = 3.74$; $n = 38$; $p = 0.0006$) (Fig. 3). Fecundity was positively related with all morphometric variables of *A. angelicus* (Table 2); the variable, which best explained the number of embryos was CW (Fig. 4). Brood mass volume varied between 11.70 mm^3 (7.25 mm CW) and 236.70 mm^3 (14.25 mm CW), and increased significantly with female CW ($t = 5.09$; $n = 47$; $p < 0.001$) (Fig. 4). Recently produced embryos

Table 1. Fecundity, embryo volume during embryogenesis (Stage I–III) and carapace width of corresponding ovigerous females of *Austinotheres angelicus* (n = 47), Golfo de Nicoya, Pacific coast of Costa Rica.

Stage	N	Fecundity				Embryo volume (mm^3)				Carapace width (mm)			
		Av	SD	Min	Max	Av	SD	Min	Max	Av	SD	Min	Max
I	35	2677	1764	550	7527	0.020	0.003	0.013	0.027	7.60	1.18	5.02	10.87
II	6	3029	2599	955	7900	0.023	0.005	0.017	0.031	7.27	1.21	5.70	9.00
III	6	4890	3176	830	8509	0.024	0.005	0.017	0.031	8.10	3.10	6.07	14.25

Table 2. Relation between morphometric features (CW, CL, AW, and AL) and fecundity in *Austinotheres angelicus* (n = 47) in the Golfo de Nicoya, Pacific coast of Costa Rica.

	CW	CL	AW	AL
R^2	0.33	0.09	0.29	0.29
T	4.69	2.13	4.27	4.25
P	<0.001	0.04	0.001	<0.001

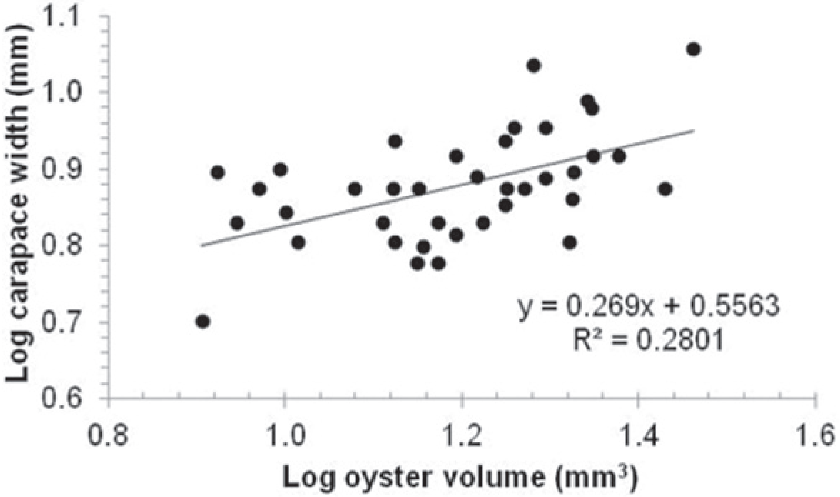

Figure 3. Relation between oyster volume (*Saccostrea palmula*) and carapace width of ovigerous females of *Austinotheres angelicus* (n = 38; exclusively females with recently produced embryos) in the Golfo de Nicoya, Pacific coast of Costa Rica.

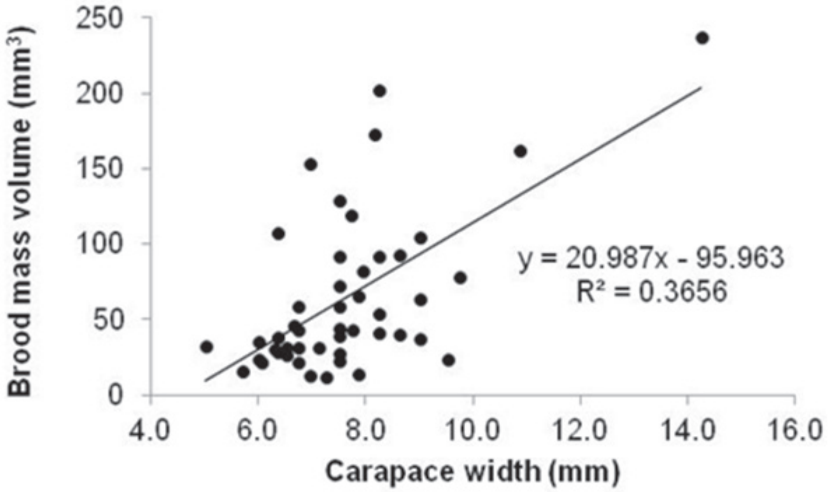

Figure 4. Relation between brood mass volume and carapace width of ovigerous females of *Austinotheres angelicus* (n = 47) in the Golfo de Nicoya, Pacific coast of Costa Rica.

(Stage I) had an average volume of 0.020 mm^3, while those close to hatching (Stage III) measured 0.024 mm^3 (Table 1); embryo volume increased during the embryogenesis by 21%, but did not vary significantly between stages ($\chi^2 = 4.51$; gl = 2; $p = 0.10$). The mean RO was 76.7 ± 28.90%, and values fluctuated between 21.7 and 162.8%.

Discussion

This is the first published report on reproductive aspects of *A. angelicus*. Cabrera Peña et al. (2001) studied the size composition and fecundity of *Juxtafabia muliniarum* (Rathbun, 1918) in the Pacific of Costa Rica; however, according to Campos and Vargas-Castillo (2014) they confused the species with *A. angelicus*. Regarding reproductive traits, Cabrera Peña et al. (2001) provided only average embryo numbers and stated that ovigerous females of this pea crab species were present throughout the study period (May 1998 to May 1999).

Fecundity

Brood size increases with female body size have been well documented for pinnotherids (Hines 1992); not surprisingly, this study has found that small-sized species such as *A. angelicus* produce less offspring than substantially larger species (Table 3). The only other published data about reproductive features of *A. angelicus* (Cabrera Peña et al. 2001; reported as *J. muliniarum*) indicate a slightly lower average fecundity than obtained in the present study (Table 3). This difference is probably related to the fact that our material contained larger females (as CW) as compared to those specimens analyzed by Cabrera Peña et al. (2001).

Brood loss

Brood loss is a well described phenomenon in decapods (for review: Kuris 1991). Our results did not reveal any embryo loss during the embryogenesis in *A. angelicus*; in fact, average embryo number in Stage I was higher than in Stage III (Table 1). This surprising result is explained by the fact that in our study specimens carrying embryos in Stage III were considerably larger than those with recently extruded embryos (Table 1). Lardies and Castilla (2001) hypothesized that the internal habitat of the host as well as the immobility of the commensal may effect reduction of embryo loss when they found low brood mortality rates in *Pinnaxodes chilensis* H. Milne Edwards, 1837, living as a commensal on a sea urchin. Our data seem to corroborate this hypothesis. However, additional studies with similar-sized females of *A. angelicus* carrying embryos in different developmental stages are necessary to answer the question whether decapods protected by its host show less or no brood mortality during embryogenesis.

Table 3. Comparison of female size (CL and CW), fecundity, embryo volume during early embryogenesis, and reproductive output of different pinnotherids; host species and study sites are indicated. NA: no data available.

Species	CL (mm)	CL range (mm)	CW (mm)	CW range (mm)	Average fecundity	Fecundity range	Embryo volume (mm^3)	RO (%)	Host	Study site	Reference
Austinixa gorei	NA	NA	6.80	6.30–8.90	NA	195–525	NA	NA	*Gilvossius setimanus* (Malacostraca, Callianassidae)	United States of America, northwest Point on Key Biscayne in Bear Cut	McDermott (2006)
*Austinotheres angelicus**	5.60	4.00–7.60	6.87	4.90–9.40	2032**	680–3300	NA	NA	*Saccostrea palmula* (Bivalvia, Ostreidae)	Costa Rica, mangroves at Punta Morales, Pacific coast	Cabrera Peña et al. (2001)
Austinotheres angelicus	5.43	3.82–9.37	7.60	5.03–10.87	2677	550–8509	0.020	77	*Saccostrea palmula* (Bivalvia, Ostreidae)	Costa Rica, Punta Morales, Pacific coast	Present study
Fabia subquadrata	NA	NA	NA	NA	7560	NA	0.037	97	*Mytilus californianus* (Bivalvia, Mytilidae)	United States of America, Bodega Head, CA	Hines (1992)
Pinnaxodes chilensis	12.22	8.20–15.50	NA	NA	4553	2134–9456	0.048	70	*Loxechinus albus* (Echinoidea, Parechinidae)	Chile, Caleta Coloso	Lardies and Castilla (2001)
Pinnaxodes chilensis	16.39	10.75–20.00	NA	NA	8358	2376–15898	0.070	80	*Loxechinus albus* (Echinoidea, Parechinidae)	Chile, El Quisco	Lardies and Castilla (2001)
Pinnaxodes chilensis	18.59	17.40–20.30	NA	NA	8082	5045–15432	0.072	81	*Loxechinus albus* (Echinoidea, Parechinidae)	Chile, Mehuín	Lardies and Castilla (2001)
Zaops ostreus	NA	NA	NA	NA	5680	NA	0.092	66	*Crassostrea virginica* (Bivalvia, Ostreidae)	United States of America, Indian River, FL	Hines (1992)

*Reported as *Juxtafabia muliniarum*;

**Stage of embryonic development used to calculate fecundity is unknown.

Embryo volume

The embryo volume of *A. angelicus* increased during the incubation period by 21%. This increase is relatively low when compared to other marine decapods living in association with other invertebrates: *Synalpheus yano* (Ríos & Duffy, 2007) in the sponge *Lissodendoryx colombiensis* Zea & van Soest, 1986; 118% (Hernáez et al. 2010); and, *Pinnaxodes chilensis* (H. Milne Edwards, 1837) in the sea urchin *Loxechinus albus* (Molina, 1782): ranging from 58 to 77% (Lardies and Castilla 2001). Lardies and Wehrtmann (1996) suggested that increased water uptake during embryogenesis might serve as a buffer against environmental changes outside the embryo. Therefore, low embryo volume increase might suggest relatively stable conditions for embryo development of *A. angelicus* in its host.

Embryo size has been considered as an indicator for energy content provided by the female (McEdward and Coulter 1987). Compared to other pinnotherids (Table 3), *A. angelicus* produced relatively small embryos, and the embryo volume (0.020 mm^3) is considerably smaller than the average value (0.045 mm^3) calculated for 35 brachyuran crab species (Hines 1992). Therefore, it is concluded that females of *A. angelicus* allocate a relatively small amount of energy per embryo. However, this low maternal investment per offspring is compensated by a relatively large brood mass, which is reflected by extraordinary high RO values (up to 162.8%). These findings corroborate results of previous studies with pea crabs (Hines 1992, Lardies and Castilla 2001), which revealed substantially higher reproductive investment compared to other free-living decapod species. According to Hines (1992), the following features might explain the high RO values in pinnotherid crabs: (1) apparently in contrast to other decapods, ovaries of pinnotherids can extend from the cephalothorax into the abdomen, thus creating additional space for egg accumulation; (2) the protected habitat allows a reduced calcification of the exoskeleton, which in turn diminishes female body weight and makes the body more flexible, allowing to distend during egg accumulation. The high RO values of pinnotherid crabs are an adaptation to its symbiotic life style (Hines 1992). Our results regarding *A. angelicus* provide further evidence that pinnotherids can produce proportionately much larger broods than free-living crabs.

Relation between host size and *Austinotheres angelicus*

The size of *A. angelicus* increased significantly with the volume of its host, *S. palmula*. This finding concurs with results reported by Sun et al. (2006) who found that CWs of the pea crab *Pinnotheres sinenis* Shen, 1932 increased with the weight of both the shell and the soft tissue of its host *Mytilus galloprovincialis*. Also, Atkins (1926) found the largest individuals of *Pinnotheres pisum* (Linnaeus, 1767) in large-sized specimens of its host mussel, *Mytilus edulis*.

Conclusions

Representatives of the family Pinnotheridae have evolved a series of adaptations to cope with their symbiotic life style (Hines 1992; Peiró and Mantelatto 2011), which makes them an interesting model to study the evolution of associations between decapods and other invertebrates. Here we present results on reproductive features of *A. angelicus*, which lives in association with oysters. Since the host plays a fundamental role in the life cycle of the pea crab, it seems necessary to get a better understanding of the interactions between population dynamics of the host and adaptive responses of the symbiotic pea crab. Hernández et al. (2012) assumed that small host populations induce pea crabs to adopt a solitary life style, and predicted that stable dwellings stimulate extended parental care. Moreover, the larval phase and recruitment processes are unknown in *A. angelicus*, and it has been speculated that pinnotherid larvae suffer higher mortality rates while searching for the specialized recruitment place (different types of hosts) than free-living species (Lardies and Castilla 2001). Finally, *A. angelicus* has been reported as symbiont from different hosts (Campos 2002), which raises the question if populations of this pea crab develop different life cycle adaptations in different hosts.

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RESEARCH ARTICLE

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Population structure, sex ratio and growth of the seabob shrimp *Xiphopenaeus kroyeri* (Decapoda, Penaeidae) from coastal waters of southern Brazil

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Abstract

This study evaluated the growth and population structure of *Xiphopenaeus kroyeri* in Babitonga Bay, southern Brazil. Monthly trawls were conducted from July 2010 through June 2011, using a shrimp boat outfitted with double-rig nets, at depths from 5 to 17 m. Differences from the expected 0.5 sex ratio were determined by applying a Binomial test. A von Bertalanffy growth model was used to estimate the individual growth, and longevity was calculated using its inverted formula. A total of 4,007 individuals were measured, including 1,106 juveniles (sexually immature) and 2,901 adults. Females predominated in the larger size classes. Males and females showed asymptotic lengths of 27.7 mm and 31.4 mm, growth constants of 0.0086 and 0.0070 per day, and longevities of 538 and 661 days, respectively. The predominance of females in larger size classes is the general rule in species of Penaeidae. The paradigm of latitudinal-effect does not appear to apply to seabob shrimp on the southern Brazilian coast, perhaps because of the small proportion of larger individuals, the occurrence of cryptic species, or the intense fishing pressure in this region. The longevity values are within the general range for species of Penaeidae. The higher estimates for longevity in populations at lower latitudes may have occurred because of the growth constants observed at these locations, resulting in overestimation of this parameter.

Keywords

Asymptotic length, von Bertalanffy, longevity, Dendrobranchiata

Introduction

The impact of shrimp fisheries in tropical regions is now comparable to impacts on the world's most intensively exploited temperate continental-shelf ecosystems. These fisheries have caused significant losses of spawning biomass and biodiversity, especially as a consequence of trawling on soft bottoms (Pauly and Christensen 1995, Pauly et al. 2002). Information about population biology can be important for understanding the life cycle of intensively fished species such as the seabob shrimp *Xiphopenaeus kroyeri* (Heller). This information can be developed from measurements of the size-class distribution, sex ratio, modal progression, growth, and longevity at spatial and temporal scales (Gab-Alla et al. 1990, Nakagaki and Pinheiro 1999).

Xiphopenaeus kroyeri has a wide geographical range in the western Atlantic Ocean, from Cape Hatteras (North Carolina, USA) to southern Brazil (Rio Grande do Sul) (Costa et al. 2007). Of the several species targeted by artisanal fishermen in southern Brazil, the seabob shrimp is one of the most important, and is also among the top ten penaeid species caught worldwide (Gillett 2008, Silva et al. 2013). Unfortunately, this species is overexploited in southeastern and southern Brazil (Vasconcellos et al. 2007, Almeida et al. 2012).

Many investigators have suggested that several environmental parameters and resources affect the observed patterns of population dynamics of species of decapod crustaceans. These parameters include temperature oscillations (proximate factor) and plankton productivity (ultimate factor), among others, and all are affected to various degrees by latitude (Bauer 1992, Clarke 1993, Boschi 1997, Castilho et al. 2007b, Costa et al. 2010).

Knowledge of the growth and longevity of penaeid shrimps is still limited, although these are important attributes in the study of population dynamics of heavily exploited vulnerable species (Petriella and Boschi 1997). The lack of studies on growth in decapod crustaceans can be attributed to difficulties in estimating growth, which are related to the absence of structures that can provide information about aging (Petriella and Boschi 1997, Branco 2005, Vogt 2012). Because growth in decapod crustaceans is discontinuous, frequently interrupted by successive ecdyses, the von Bertalanffy (1938) model is the most useful for the study of animals that grow rapidly, such as penaeid and sergestid shrimps (Petriella and Boschi 1997).

The present study evaluated the population biology of *X. kroyeri* in the Babitonga Bay region, focusing on the sex ratio at different times of the year, juvenile recruitment, growth rates, and longevity of males and females. The longevity of *X. kroyeri* was compared with studies of the same species at different latitudes, to determine whether the latitudinal paradigm is applicable to this population.

Methods

Study area

The sampling area of the present study is located in a subtropical region known as the Atlantic upwelling zone (from 23°S to 29°S). In the Atlantic Ocean, open ocean circulation is dominated by the opposing flow of the Brazil (subtropical) and the Malvinas (subantarctic) currents, which meet on average at 36°S (Acha et al. 2004). In southern Brazil, Babitonga Bay has an estuarine area surrounded by mangrove forests, and an adjacent marine environment with a high conservation priority (MMA 2007), and is a leading candidate for the creation of a sustainable-use marine protected area (MPA) (Vilar et al. 2011). The bottom sediment in the bay is composed mainly of sand, silty sand, and sandy silt, with little salinity stratification (IBAMA 1998). Tides are mixed and predominantly semi-diurnal (IBAMA 1998). During winter, the water temperature is vertically homogeneous, and during summer the cold water mass termed the South Atlantic Central Water (SACW) influences the region (Marafon-Almeida et al. 2008). The local fishing villages harvest crabs, shrimps, oysters, clams and fish, using gillnets, bottom trawling, long-lines, cast nets and the gerival (a tide/motor-driven net that targets shrimp) (IBAMA 1998, Pinheiro and Cremer 2003). The local fauna is poorly studied, particularly the seabob shrimp *X. kroyeri*, despite its socioeconomic importance as attested by many investigators (Rodrigues 2000, Pinheiro and Cremer 2003, Branco 2005, Bail and Branco 2007).

Biological sampling

Monthly trawls were conducted in the ocean adjacent to Babitonga Bay, off the municipalities of São Francisco do Sul and Itapoá on the northern coast of the state of Santa Catarina, using an artisanal shrimp fishing boat outfitted with double-rig nets (mesh size: 3 cm; total mesh gap: 11.5 m; boat velocity during trawls: 1.6 knots; total distance traveled during trawls: approximately 0.5 miles). Trawls were performed at five different depths (5, 8, 11, 14 and 17 m), sampling for 30 min at each depth, monthly, from July 2010 through June 2011 (five trawls per day, totaling 60 trawls in the year) (Fig. 1). For each point, latitude and longitude were taken with a GPS (Garmin GPSmap 76CSx), and depth was measured with an echobathymeter.

The carapace length (CL, to the nearest 1.0 mm), used as the standard measurement, includes the distance between the posterior margin of the eye orbit and the posterior margin of the carapace, and is widely used in studies of penaeid shrimps (Castilho et al. 2007ab, 2008a, 2012, Heckler et al. 2013). Size-frequency distributions were constructed using size classes of 1 mm CL.

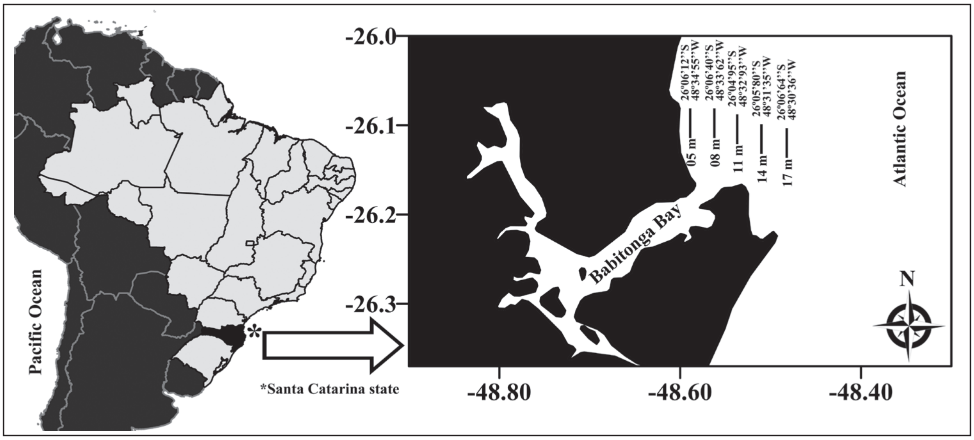

Figure 1. Map of the study area, Babitonga Bay, southern Brazil (Santa Catarina state), indicating locations and depths of the sampling sites.

Sex ratio

The sex of individuals was determined by the presence (males) or absence (females) of petasma. The sex ratio was estimated as the quotient between the number of males and the total number of individuals in samples from each month. Deviations from a 1:1 sex ratio were tested using a binomial test ($\alpha = 0.05$) (Wilson and Hardy 2002, Baeza et al. 2013). After the sexes were sorted, individuals were classified as juvenile (sexually immature) or adult. Juvenile females were considered as those with ovaries ranging from thin to thicker, transparent strands; adult females were categorized by the color and volume occupied by the gonads (Castilho et al. 2008a). Adult males were identified by their fused petasma, and juvenile males by separated petasma (Boschi 1989).

Individual growth and longevity

Growth and longevity were analyzed for males and females separately (Boschi 1969), based on the von Bertalanffy growth model (von Bertalanffy 1938) and using the methodology adopted by Simões et al. (2013). Modal values were determined for each CL frequency using the software PEAKFIT (Automatic Peak Fitting Detection and Fitting, Method I-Residual, no Data Smoothing), with size classes of 1.0 mm, according to Fonseca and D'Incao (2003). The models were plotted on a scatter graph vs. age, to analyze the growth rhythm of the cohorts. Growth parameters (CL_∞: asymptotic carapace length; k: growth coefficient (day^{-1}); t_0: theoretical age at size zero) were estimated by using the SOLVER supplement in Microsoft Excel (version 2010) for Windows 7, which applies the Von Bertalanffy growth model: $CL_t = CL_\infty[1\text{-exp}^{-k(t-t0)}]$ (CL_t: carapace length at age t). The growth of a cohort was evaluated based on its similarity to values

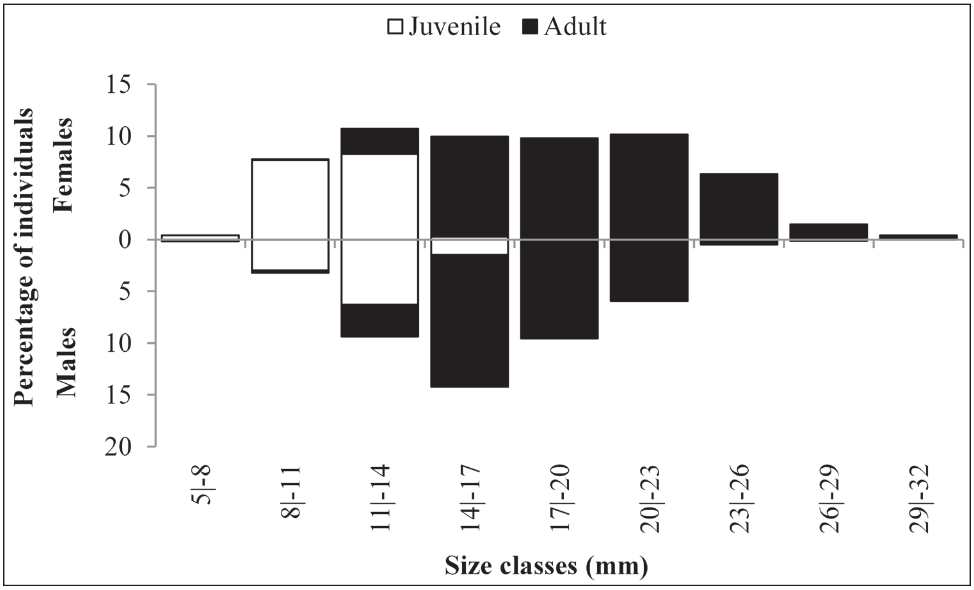

Figure 2. *Xiphopenaeus kroyeri*. Distribution of the percentage of juveniles and adults by size classes (CL, mm) observed for individuals collected from July 2010 through June 2011 in an area adjacent to Babitonga Bay, southern Brazil.

previously observed for this species (Campos et al. 2011, Heckler et al. 2013). Cohort data were pooled and growth parameters were estimated. The estimated growth curves for males and females were compared by F test (p=0.05) (Cerrato 1990). Longevity was calculated using the inverse von Bertalanffy growth model, with a modification suggested by D'Incao and Fonseca (1999), which is given by: longevity= $0-(1/k)\mathrm{Ln}[1-(CL_t/CL_\infty)]$ (considering t_0= 0, and CL_t/CL_∞= 0.99).

Results

Population structure

Throughout the sampling period, 4,007 specimens of *X. kroyeri* were examined, including 1,722 males and 2,285 females (43% and 57%, respectively) of which 2,901 were adults and 1,106 juveniles. Carapace length ranged from 7.1 to 29.7 mm for males and 6.0 to 31.8 mm for females.

Among adults, males were more abundant in the size classes between 8.0 and 16.9 mm. Beginning in the 17.0–19.9 mm size class, adult females were more abundant, with a peak in the 20.0–22.9 mm size class. Juvenile individuals occupied the lower size classes, predominating in the 11.0 to 13.9 mm size class; and were not found in size classes larger than 17.0–20.0 mm (Fig. 2).

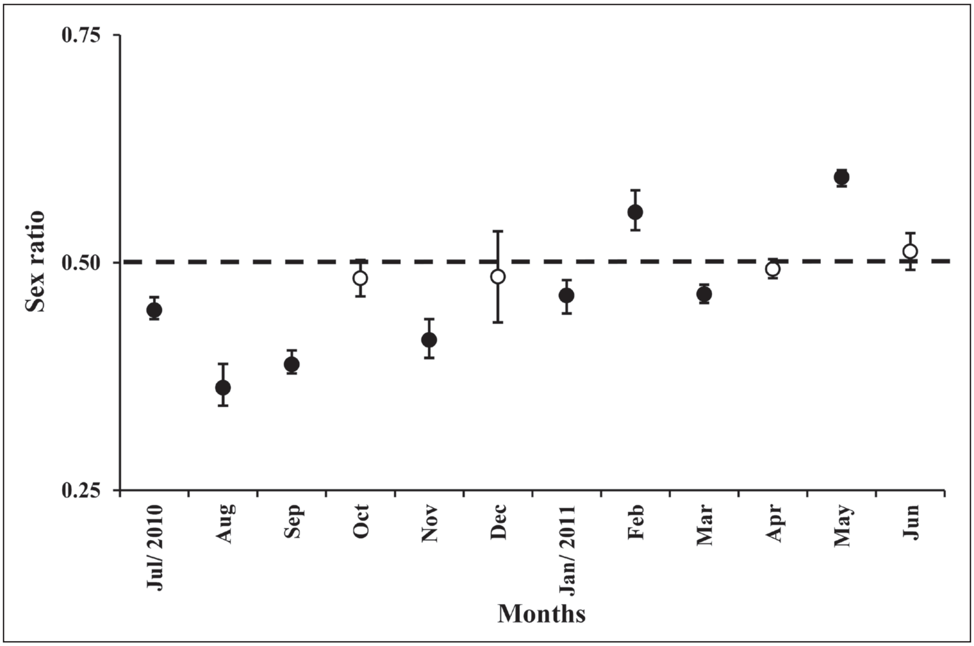

Figure 3. *Xiphopenaeus kroyeri*. Monthly sex ratio (estimate ± standard error) of adults collected from July 2010 through June 2011 in an area adjacent to Babitonga Bay, southern Brazil. Black circles indicate significant deviation from a 1:1 sex ratio (Binomial test, $p < 0.05$).

Sex ratio

During the study period, we observed a mean sex ratio of 0.5. Female-biased sex ratios (<0.5) were observed in July, August, September, November, January and March, while male-biased sex ratios (>0.5) were obtained in February and May (Fig. 3).

Individual growth and longevity

Based on the modal values, 13 cohorts for males (Fig. 4) and 11 cohorts for females (Fig. 5) were selected. An overall mean growth curve was constructed, grouping the cohorts obtained for males and females separately (Figs 6, 7). Based on these curves, overall growth models were determined for each gender, which resulted in estimates of CL_∞=27.73 mm, k = 0.0086/day (3.14/year) and t_0= -0.035 for males and CL_∞= 31.41 mm, k = 0.0070/day (2.56/year) and t_0= -0.25 for females.

Longevity was estimated to be 538 days (or 1.47 years) for males and 661 days (or 1.81 years) for females. Growth curves for males and females showed a significant difference ($F_{calculated}$= 3.157 > $F_{critical}$= 2.695; p = 0.028; degrees of freedom: 3/101).

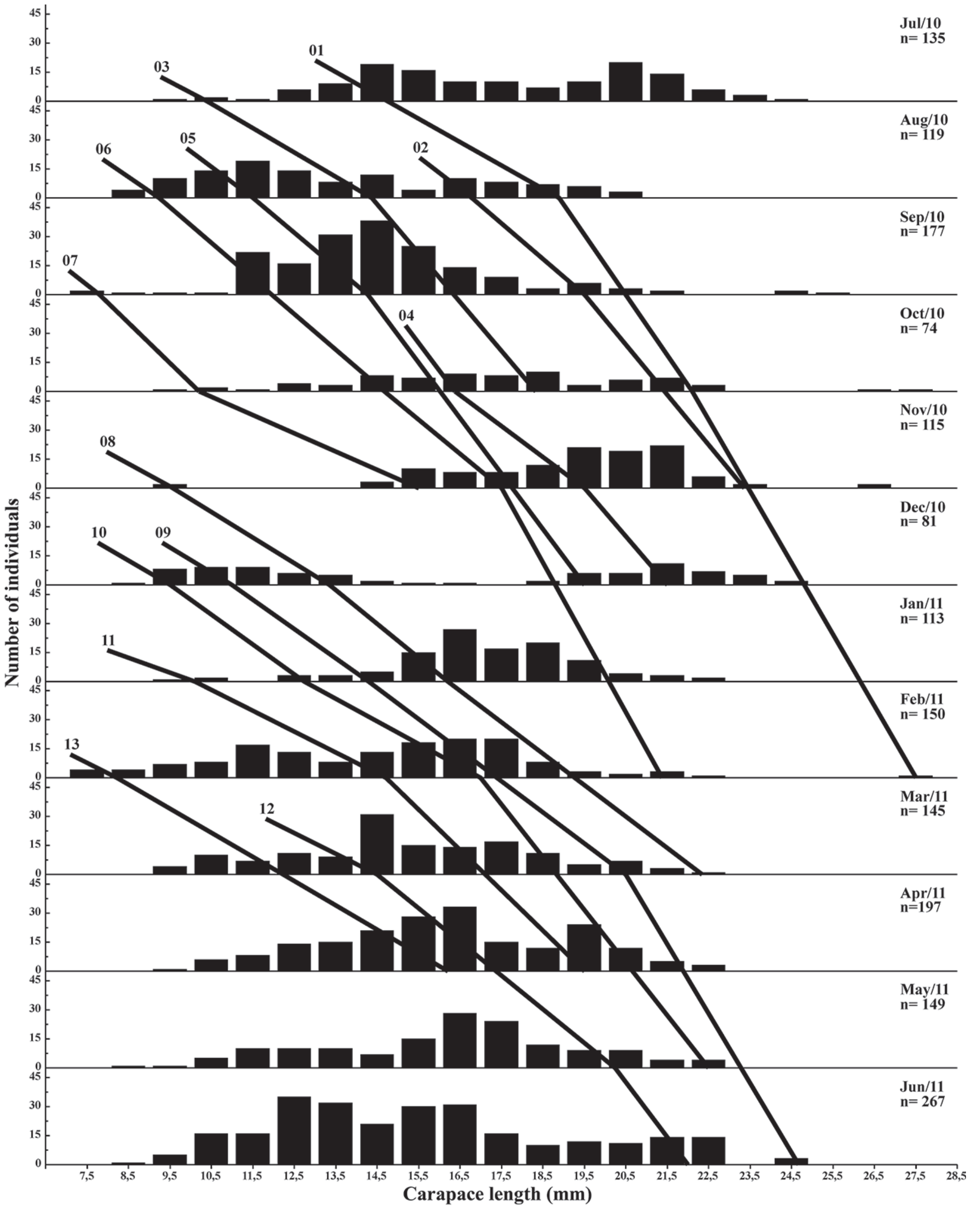

Figure 4. *Xiphopenaeus kroyeri*. Selected cohorts for growth analysis and number of males collected each month from July 2010 through June 2011 in an area adjacent to Babitonga Bay, southern Brazil.

Discussion

For Dendrobranchiata, results of several studies have revealed a predominance of females in higher size classes, e.g., for *Artemesia longinaris* Bate (Castilho et al. 2007a, Costa et al. 2010), *Pleoticus muelleri* (Bate) (Castilho et al. 2008a, Dumont and D'Incao

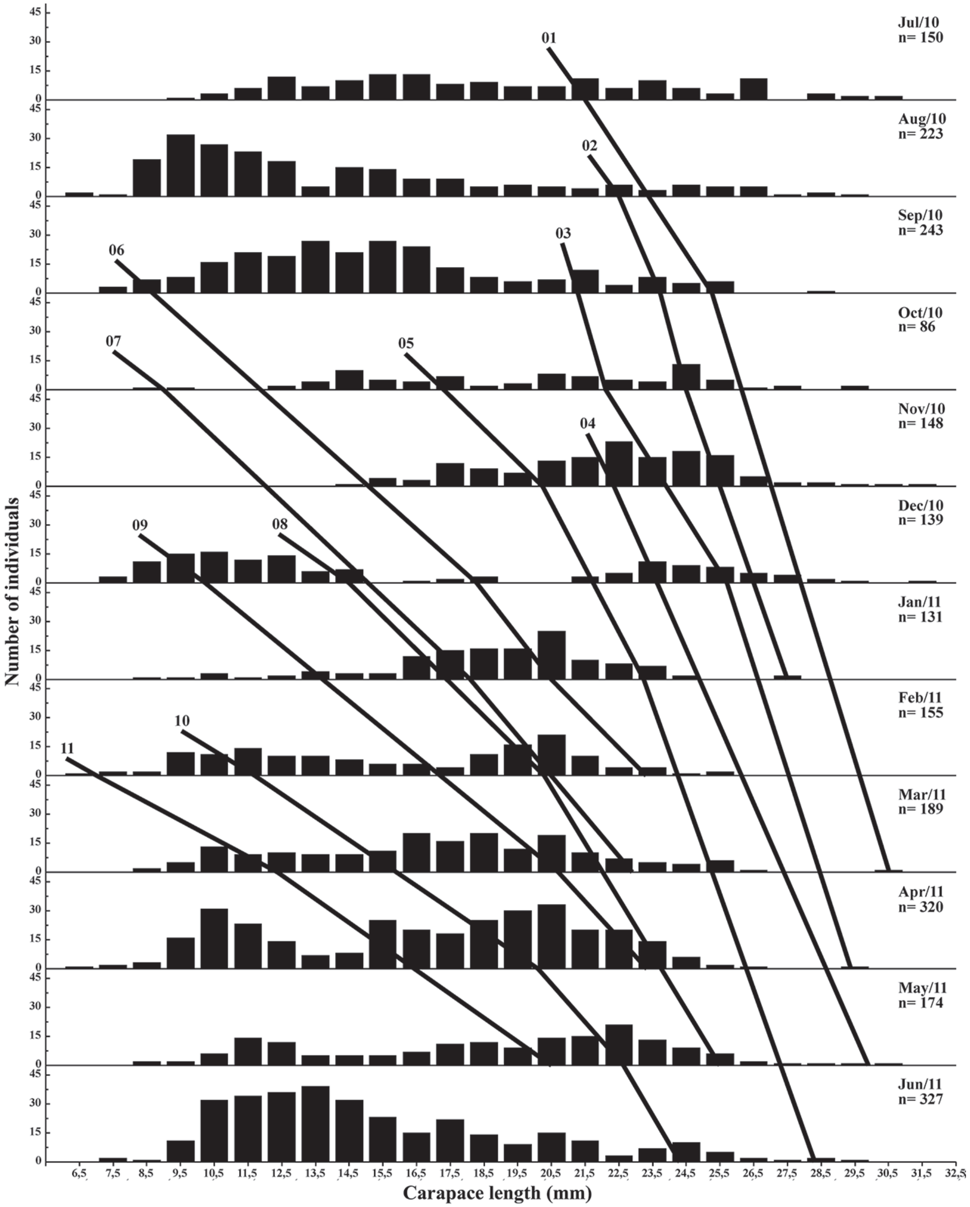

Figure 5. *Xiphopenaeus kroyeri*. Selected cohorts for growth analysis and number of females collected each month from July 2010 through June 2011 in an area adjacent to Babitonga Bay, southern Brazil.

2008) and *Sicyonia dorsalis* Kingsley (Castilho et al. 2008b), as well as for *X. kroyeri* (Branco 2005, Castro et al. 2005, Almeida et al. 2012, Eutrópio et al. 2013, Heckler et al. 2013). These studies also collected overall more females than males, which concords with results of the present study. In penaeid shrimps, females generally reach larger sizes than males (Boschi 1969, 1989). As observed by Gab-Alla et al. (1990), Branco et

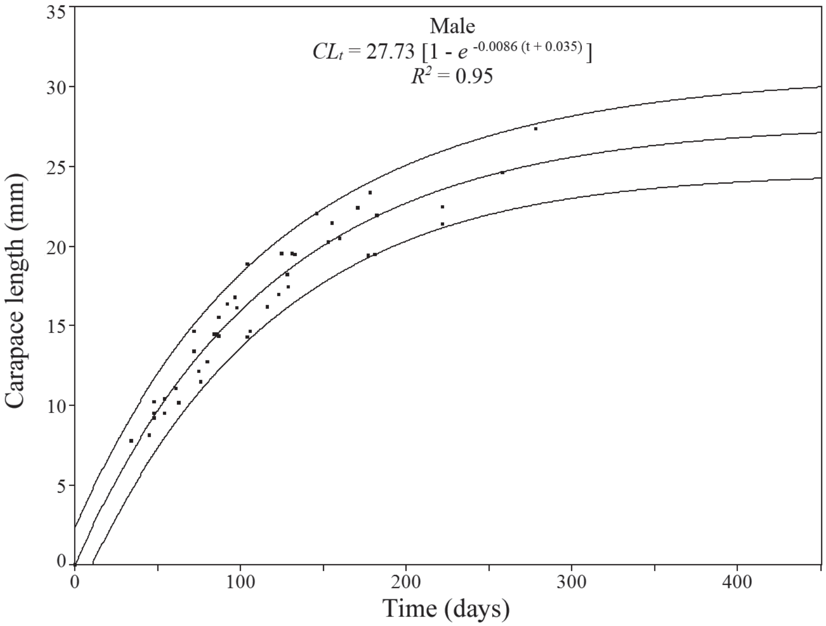

Figure 6. *Xiphopenaeus kroyeri*. Mean growth curve estimated for males collected in an area adjacent to Babitonga Bay, from July 2010 through June 2011, based on the von Bertalanffy growth model. Outer lines: 95% prediction interval.

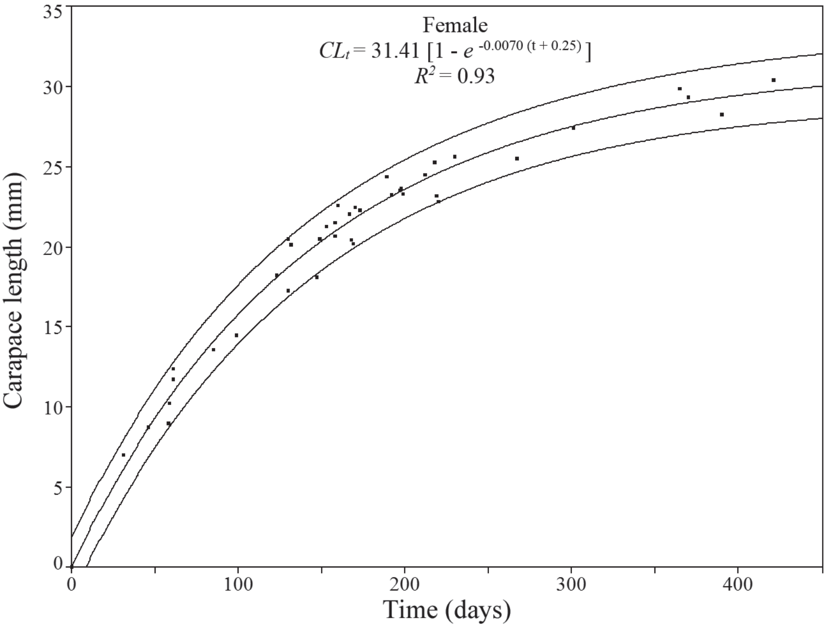

Figure 7. *Xiphopenaeus kroyeri*. Mean growth curve estimated for females collected in an area adjacent to Babitonga Bay, from July 2010 through June 2011, based on the von Bertalanffy growth model. Outer lines: 95% prediction interval.

al. (1999) and Yamada et al. (2007), this difference may be associated with the reproductive biology, as an increase in carapace length indicates a capacity to produce more oocytes, with a consequent increase in fecundity.

Several studies regarding *X. kroyeri* (Branco et al. 1994, Nakagaki and Negreiros-Fransozo 1998, Lopes et al. 2010) reported male-biased sex ratios. These fluctuations can be attributed to segregated distributions of the sexes at certain times of year (Signoret 1974, Branco 2005). Alternatively, a female-biased sex ratio may be associated with higher mortality in males (Cha et al. 2002). Kevrekidis and Thessalou-Legaki (2006), studying *Melicertus kerathurus* (Forskål), stated that the high metabolic demand of females during the period of gonadal maturation forces them to feed for long periods, increasing their vulnerability to sampling gear. Lopes et al. (2010) stated that a male-biased sex ratio is less common in *X. kroyeri*, and might be associated with migration of females to greater depths during spawning periods.

Gender is among the factors that influence growth in Penaeoidea (Petriella and Boschi 1997). Generally, males of Penaeidae show higher growth coefficients than females, resulting in lower asymptotic lengths, and males are smaller than females of equal age. In this study, females showed a lower growth coefficient and greater asymptotic length, which concords with information provided by García and Le Reste (1986) and Petriella and Boschi (1997), as well as with previous analyses of the growth of this species along the Brazilian coast (Branco 2005, Fernandes et al. 2011, Heckler et al. 2013).

On the Brazilian coast, the growth of *X. kroyeri* has been studied by Santos and Ivo (2000) on the coast of the state of Bahia; Heckler et al. (2013) off São Paulo; and Campos et al. (2011) off Santa Catarina, among others. Santos and Ivo (2000) obtained the lowest k values, as well as the highest asymptotic length (females: k = 0.767/year, CL_{∞}= 37.2 mm; males: k = 0.986/year, CL_{∞}= 28.0 mm), followed by Campos et al. (2011) (females: k = 2.628/year, CL_{∞}= 31.0 mm; males: k = 2.993/year, CL_{∞}= 28.0 mm) and by Heckler et al. (2013) (females: k = 2.190/year, CL_{∞}= 30.54 mm; males: k = 3.280/year, CL_{∞}= 26.38 mm). Castilho et al. (2007b) suggested that several aspects of the life cycle such as longevity, body size, and size at the onset of sexual maturity show variations related to environmental factors that can be associated with latitude. As latitude increases, individuals tend to grow larger and more slowly, probably because of the colder water temperatures. This is known as the latitudinal effect paradigm, which has been analyzed with respect to its influence on population dynamics of penaeid shrimps by several researchers (Bauer 1992, Boschi 1997, Costa and Fransozo 2004, Castilho et al. 2007b, Costa et al. 2010).

The results of growth analyses for *X. kroyeri* along the southern Brazilian coast do not seem to follow the latitudinal effect pattern. The lower growth coefficient (and consequently the greater asymptotic length) observed by Santos and Ivo (2000) can be explained by the methodology adopted by the authors, who used the software FAO-ICLARM Stock Assessment Tool (FISAT), which includes the ELEFAN routine (Electronic Length Frequency Analysis). Fonseca (1998) noted that this software tends to exclude extreme lengths from the analysis, which causes errors in estimating the growth coefficient. The analysis conducted by Freire (2005) on the São Paulo coast

(Ubatuba) provided higher results for asymptotic length, compared to that obtained by Campos et al. (2011) on the Santa Catarina coast and to the present results. These differences can be explained by differences in sampling depth, since the study by Freire (2005) included deeper sites (up to 35 m). The results obtained in the present growth analyses are consistent with the maximum size of the shrimp observed in field sampling (females: estimate = 31.41 mm, field observation = 31.80 mm; males: estimate = 27.73 mm; field observation = 27.90 mm).

Three additional hypotheses may explain the disagreement here presented in relation to the latitudinal effect pattern. First, as observed by Gusmão et al. (2006), it is possible that two different species of *Xiphopenaeus* live in the southwestern Atlantic. In the brazilian coast, these authors examined an area extending from Natal in the state of Rio Grande do Norte in northeastern Brazil, to Ubatuba, and found that *Xiphopenaeus* sp. 1 occurred throughout the area, whereas *Xiphopenaeus* sp. 2 showed a discontinuous distribution, with recorded occurrences only at Natal and Ubatuba. The second hypothesis is based on the number of individuals sampled in higher size classes, which can be explained in many ways. As noted by Campos (2006), larger animals could be less vulnerable to capture, and may have some ability to escape from the sampling gear. The author also suggested that larger individuals migrate to areas that are not accessible to the artisanal fishery. In agreement with this suggestion, Freire (2005) observed an increasing trend in CL toward greater depths, explaining the higher CL values found in his study. In further support of the difference in efficiency of the fishing methods, in 2011, even with a smaller fleet (14% of 806 vessels), the industrial fishery accounted for 47% of all seabob shrimp landings in the state of São Paulo (Mendonça et al. 2013).

The third hypothesis considers the intense fishery effort off the coast of Santa Catarina, which has been studied by D'Incao et al. (2002), Branco (2005) and Vasconcellos et al. (2007), among others. Although Mendonça et al. (2013) reported that the seabob shrimp stocks off São Paulo state have stabilized in recent years, Vasconcellos et al. (2007) argued that these stocks off southeastern and southern Brazil are overexploited. This intense fishery exploitation was responsible for the decrease in yield of the seabob shrimp fishery in these areas (D'Incao et al. 2002). It is entirely possible that, due to the intense fishery effort in this area, the stocks cannot maintain their normal population structure, i.e., individuals cannot reach larger size classes.

The absence of a pattern of asymptotic length estimated for this species may be a consequence of gene flow between the populations off Santa Catarina and São Paulo, since under favorable conditions the shrimp can migrate up to 900 km, as observed by Fenucci (1988) for *Melicertus plebejus* (Hess) on the Australian coast. Also, similarities in reproductive biology among populations that are in some cases located thousands of kilometers apart, may indicate the existence of an open meta-population, with considerable connectivity in the southwestern Atlantic (Almeida et al. 2012).

The present results for the longevity and growth coefficient of *X. kroyeri* fall within the range proposed by García and Le Reste (1986) and Pauly (1984) for these parameters in penaeid shrimps. These investigators found that the shrimp complete their life cycle in approximately two years, with growth coefficients between 0.25 and 2.5. Our estimates suggest that, the seabob shrimp in the study area has a longevity of 538 days or 1.47 years for males, and 661 days or 1.81 years for females. Similarly, Campos et al. (2011) on the coast of Tijucas, Santa Catarina, estimated a longevity of 561 days for males and 641 days for females. Analyses of populations at lower latitudes have estimated greater longevities for males and females respectively: 1705 and 2192 days (Santos and Ivo 2000), 2192 and 2422 days (Santos 1997) and 1535 and 1212 days (Santos 1997). According to D'Incao and Fonseca (1999), most longevity analyses for penaeid shrimps have resulted in overestimates; based on underestimates for the growth coefficient, which increases the longevity estimate.

Conclusion

In general, the present results indicate that *X. kroyeri* completes its life cycle within the study area, because juveniles as well as adults with a range of sizes were collected. All estimates in our study concord with current knowledge of the life cycle of *X. kroyeri*, are within the ranges proposed by several investigators, and are similar to values observed in field sampling. This study provides a theoretical basis for informed management of this fishery along the Brazilian coast.

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RESEARCH ARTICLE

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Inferring population connectivity across the range of distribution of the stiletto shrimp *Artemesia longinaris* Spence Bate, 1888 (Decapoda, Penaeidae) from DNA barcoding: implications for fishery management

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Abstract

Artemesia longinaris is a marine shrimp endemic to the southwestern Atlantic and distributed from Atafona, Rio de Janeiro (Brazil) to Rawson, Chubut (Argentina). In recent years, this species has become an important target of the commercial fishery as a consequence of the decline in the fishery of more traditional and profitable marine shrimps. In addition, phenotypic variations have been documented in populations along its distribution. Therefore, investigations on the genetics of the fishing stocks are necessary for the development of sustainable management strategies and for understanding the possible sources of these variations. The mitochondrial gene Cytochrome Oxidase I (COI) was used to search for evidence of genetic structure among the populations of *A. longinaris* and to analyze the phylogenetic relationships among them. A total of 60 specimens were collected from seven different localities, covering its geographical range. The final alignment showed 53 haplotypes (48 individuals and 5 shared), with no biogeographical pattern. The low genetic divergence found, with a non-significant FST value, also suggests the absence of population structure for this gene. These findings indicate a continuous gene flow among the populations analyzed, suggesting that the phenotypic variation is a consequence of different environmental conditions among the localities.

Keywords
Cytochrome Oxidase I, gene flow, Penaeoidea, phenotypic plasticity

Introduction

Artemesia longinaris Spence Bate, popularly known as Argentine stiletto shrimp, plays an important role in the marine trophic chain of the southwestern Atlantic, as food for different species of fish and cephalopods (Capitoli et al. 1994). In recent years, however, this species has become a common target of both artisanal and industrial fisheries. The former occurs along its entire distribution and the latter is mainly concentrated in southern Brazil and Argentina (D'Incao et al. 2002). The increase in the fishery of *A. longinaris* is a consequence of a decline in the stocks of more traditional and profitable marine shrimps, such as the pink shrimp *Farfantepenaeus brasiliensis* (Latreille) and *F. paulensis* (Pérez-Farfante), the white shrimp *Litopenaeus schmitti* (Burkenroad) and the seabob shrimp *Xiphopenaeus kroyeri* (Heller) (D'Incao et al. 2002, Costa et al. 2004, Carvalho-Batista et al. 2011).

In the last decades, catches in the states of south and southeast Brazil have reached thousands of tons (D'Incao et al. 2002). Furthermore, in spite of the increase in its exploitation in recent years, there is no specific management plan for *A. longinaris* in Brazil. The offseason in south and southeast coast of this country for this species and other commercial shrimps is based on the period of juvenile recruitment of *Farfantepenaeus* species, without taking account the possibility of the existence of more than one stock for these species (Franco et al. 2009).

Artemesia longinaris has a distribution restricted to the southwestern Atlantic, from Atafona (Rio de Janeiro, Brazil, 21°37'S) to Rawson (Chubut, Argentina, 43°18'S) (D´Incao 1999). Although its distribution is limited to the Argentinean biogeographical province, much of its extent (23° to 35°S) is considered a transitional region because of current mixing; this process leads to the formation of water masses with tropical and subantarctic characteristics (Boschi 2000). In addition, the northern boundary of its distribution is located in the region of Cabo Frio (Rio de Janeiro, Brazil), where there is a strong influence of upwelling events, driven by the winds and coastal topography (Acha et al. 2004).

Consequently, environmental conditions differ considerably throughout the range of *A. longinaris*. For example, in the Ubatuba region (São Paulo, Brazil) the temperature (16–30 °C) and salinity (28–38) vary widely because of the intrusion of different water masses (Fransozo et al. 2004, Costa et al. 2005); whereas near Cabo Frio (Rio de Janeiro, Brazil) the water temperature is about 20 °C and the salinity is high (>37) during most of the year (Sancinetti 2011); and on Mar del Plata coast (Buenos Aires, Argentina) the temperature varies seasonally, from 6 to 17 °C, and the salinity is slightly greater than 30 (Petriella and Bridi 1992, Guerrero et al. 1997, Acha et al. 2003).

In addition, phenotypic variations among *A. longinaris* populations have been noted. The body size and the mean size at sexual maturity ($CL_{50\%}$) increase with the latitude, from Ubatuba (São Paulo, Brazil) to Mar del Plata (Buenos Aires, Argentina), but decrease with latitude from the Farol de São Tome (Rio de Janeiro, Brazil) to Ubatuba (Boschi 1969a, Ruffino and Castello 1992, Castilho et al. 2007b, Semensato and Di Beneditto 2008, Costa et al. 2010). Differences in certain morphometric relationships have also been detected (Dumont and D'Incao 2010), as well as in the reproductive period, which tends to be continuous in lower latitudes and seasonal in higher latitudes (Christiansen and Scelzo 1971, Petriella and Bridi 1992, Castilho et al. 2007a).

In view of these environmental variations, Nascimento (1983) proposed that the populations off southern Brazil and northern Argentina are likely separated, based on the differences in their environmental preferences. However, an analysis of enzyme polymorphisms provided no support for this proposition (Weber et al. 1993). Further studies to investigate the possibility of genetic structure and covering the entire distribution of *A. longinaris* were still lacking.

Knowledge of the genetic structure of populations is important for the development and success of strategies for sustainable long-term management of fishery resources (Hillis et al. 1996). Mitochondrial DNA has been an important tool for these investigations, for terrestrial as well as aquatic organisms (Avise 1994). Among the mitochondrial molecular markers, the Cytochrome Oxidase I (COI) gene has been successfully employed to detect population structures in many species of Decapoda (Schubart and Huber 2006, Aoki et al. 2012, De Croos and Pálsoon 2012, Terossi and Mantelatto 2012). This property, together with other characteristics, has resulted in the choice of this gene as the standard marker for animal identification in the DNA barcoding technique (Hebert et al. 2003).

This study had the following aims: to evaluate the hypothesis of genetic structure among the populations of *A. longinaris;* investigate their phylogenetic relationships; and detect, if possible, evidences of speciation. To achieve these purposes, we used a partial sequence of the mitochondrial COI gene as the molecular marker. The population concept adopted was proposed by Roughgarden et al. (1989) and Krebs (1994). According to them, a population is a group of organisms of the same species that occupy the same place at a certain time. Our findings provide an appropriate theoretical basis for the development of management strategies for this fishery resource, as well as help to understand the origin of the phenotypic differences among populations of this species.

Methods

Sample collection

The specimens were obtained, at scientific cruises, from seven localities in the southwestern Atlantic (Table 1 and Fig. 1). The specimens were identified based on Costa et al. (2003), and were immediately preserved in 80% ethanol and deposited in the Crustacean Collection of the Department of Biology (CCDB), Faculty of Philosophy, Sciences and Letters at Ribeirão Preto (FFCLRP), University of São Paulo (USP) (Table 1).

Table 1. List of specimens used for molecular analysis with respective site of collection, catalogue numbers, and GenBank accession numbers for *Artemesia longinaris*. The letters CCDB preceding the catalogue numbers represent the Crustacean Collection of the Department of Biology, Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, University of São Paulo.

Locality	Catalogue numbers	GenBank Accession Numbers
Macaé-Rio de Janeiro, Brazil (22°23'44"S; 41°44'57"W)	CCDB 3782	KF572060–KF572069
Ubatuba-São Paulo, Brazil (23°27'24"S; 45°01'20"W)	CCDB 3806, 3429	KF572070–KF572082
Santos-São Paulo, Brazil (24°03'59"S; 46°16'57"W)	CCDB 4008	KF572083–KF572084
Cananéia-São Paulo, Brazil (25°08'15"S; 47°50'40"W)	CCDB 3655	KF572085–KF572089
São Francisco do Sul-Santa Catarina, Brazil (26°05'52"S; 48°33'82"W)	CCDB 3851	KF572090–KF572098
Rio Grande-Rio Grande do Sul, Brazil (32°10'23"S; 52°06'10"W)	CCDB 3928	KF572099–KF572108
Mar del Plata-Buenos Aires, Argentina (37°58'57"S; 57°32'15"W)	CCDB 869, 4150	KF572109–KF572119

DNA extraction, PCR amplification, purification and sequencing

The protocols for DNA extraction, amplification and sequencing followed Mantelatto et al. (2009) and Pileggi and Mantelatto (2010).

An ~700-bp region of a partial sequence of the mitochondrial COI gene was amplified by the polymerase chain reaction (PCR) using the pair of primers: HCO1 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al. 1994). The PCR reaction was performed in an Applied Biosystems Veriti® 96-well thermocycler, using the following thermal cycle: initial denaturing for 2 min at 94 °C followed by 35 denaturing cycles at 94 °C for 30 s, primer annealing at 50–58 °C for 30 s and extension at 72 °C for 1 min, and a final extension for 5 min at 72 °C. The PCR products were purified using the SureClean Plus® purification kit (Bioline) and were sequenced with the Big Dye® Terminator Cycle Sequencing kit in an ABI 3100 Genetic Analyzer® (Applied Biosystems Life Technologies). All sequences were confirmed by sequencing both strands.

Data analysis

The editing and construction of a consensus sequence for the two strands were conducted using the computational program BIOEDIT 7.3.1.0 (Hall 1999). Sequences were aligned using the program CLUSTAL W (Thompson et al. 1994), with interface to BIOEDIT (Hall 1999) using default parameters. The computational program MEGA 5.0 (Tamura et al. 2011) was used to estimate the average nucleotide composition and

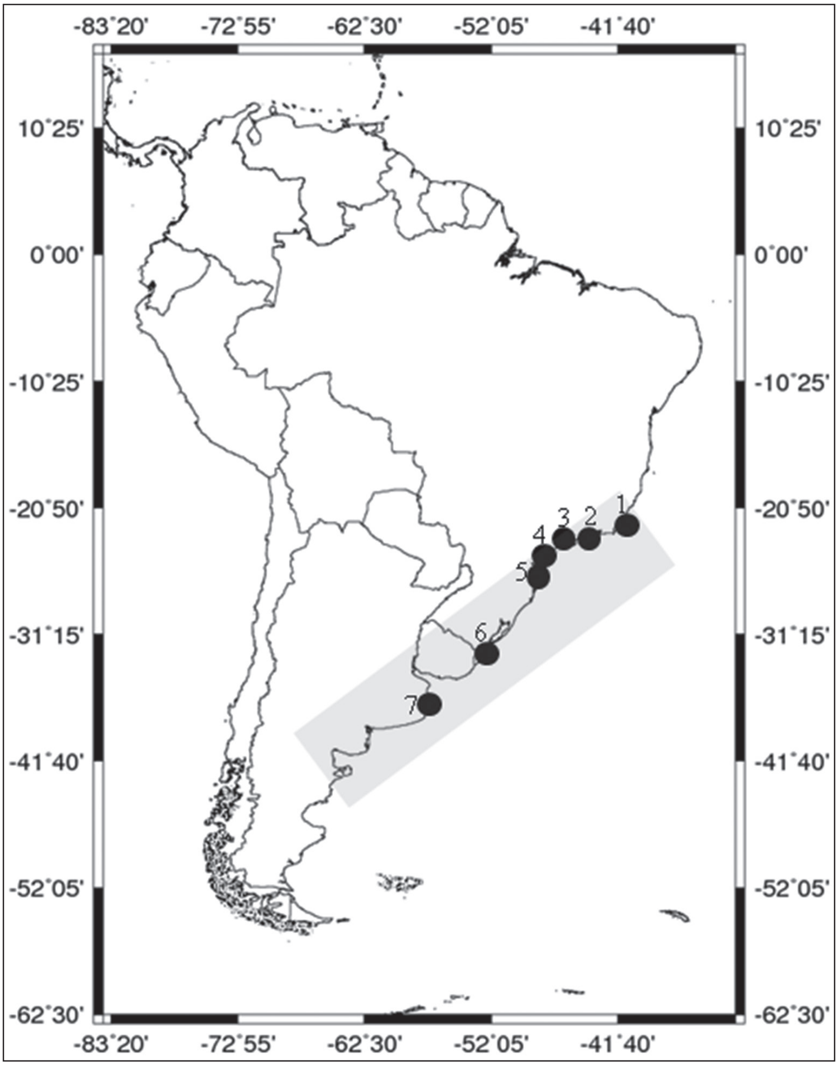

Figure 1. Southwest Atlantic collection sites. Map showing the localities of the specimens of *Artemesia longinaris* analyzed: **1** Macaé, Brazil **2** Ubatuba, Brazil **3** Santos, Brazil **4** Cananéia, Brazil **5** São Francisco do Sul, Brazil **6** Rio Grande, Brazil **7** Mar del Plata, Argentina. The gray band indicates the complete geographical distribution of *Artemesia longinaris*.

genetic distances, and to construct a Neighbor-Joining dendrogram, both based on the Kimura 2-parameter substitution model (Kimura 1980). The phylogram using the Maximum Likelihood criterion was constructed in the program RAxML-HPC2 on X-SEDE (Stamatakis 2006) through the online version of the Cyber Infrastructure for Phylogenetic Research (CIPRES) website (Stamatakis et al. 2008, Miller et al. 2010). The default parameters of RAxML were used to perform the analysis for the GTR model. To measure the consistency of the topology, we selected the option to automatically determine the number of bootstraps to be run in RAxML. Consequently, 1000 bootstrap pseudo-replicates were run, and only the values >50% were reported.

For both the genetic distance and phylogenetic analyses, sequences of three other penaeid species were included in the alignment as an outgroup: *F. brasiliensis*, *F. paulensis* (GenBank accession numbers KF783861–KF783862) and *Rimapenaeus constrictus* (Stimpson) (GenBank accession number KF783863). We also attempted to use a sequence of the same portion of the COI gene of *A. longinaris* available in GenBank (accession number EU400383.1) (Dumont et al. 2009). However, it was not possible to obtain alignments without gaps when this sequence was included. This observation, allied to the fact that its translation to an amino-acid sequence showed the presence of stop codons, indicates that this sequence must be reviewed. The presence of stop codons in the middle of an encoding gene suggests the possibility of the amplification and sequencing of a pseudogene (Buhay 2009).

The haplotype number was calculated in the program DNASP 4.10.9 (Rozas and Rozas 1999). The haplotype network was constructed by the Median-Joining method in NETWORK software (Bandelt et al. 1999), with data preparation in DNASP. The haplotype and nucleotide diversities were calculated for each locality using ARLEQUIN Version 3.1 (Excoffier et al. 2005). The genetic variation was analyzed with a analysis of molecular variance (AMOVA) (Excoffier et al. 1992), and was computed in ARLEQUIN Version 3.1 (Excoffier et al. 2005).

Results

A total of 60 sequences of the COI gene from individuals sampled in the seven localities was obtained. The final multiple sequence alignment included 645 base pairs. The number of variable sites was 66 (10.23%), 8 (12.12%) in the first codon position and 58 (87.88%) in the third position, and 30 of the variable sites were phylogenetically informative. Adding three species as the outgroup, the number of variable sites was 143 (28.49%), 72 of which were phylogenetically informative. The average nucleotide composition for *A. longinaris* was 28.41% (A), 30.99% (T), 19.47% (G), and 21.12% (C).

The intraspecific genetic distance of *A. longinaris* ranged from 0 to 2.7%, and the average distance was 1.1 ± 0.2%. The interspecific genetic distance, including the outgroup, ranged from 21.3 to 27.1%. Average distance among individuals in each population ranged from 0.81 ± 0.25% at Cananéia to 1.42 ± 0.24% at Macaé (Table 2). Among localities, distances ranged from 0.8 ± 0.2% between Santos and Cananéia to 1.4 ± 0.2% between Macaé and São Francisco do Sul (Table 3).

Both the Neighbor-Joining and Maximum Likelihood analysis indicated no structure by localities (Figs 2 and 3).

Based on the 60 sequences, 53 haplotypes were identified. Of these, 48 represented single individuals. The locality of Santos was not included in the analysis of haplotype, nucleotide diversity and molecular variance (Tables 4 and 5), since only two sequenc-

Table 2. Average distance (%) among *Artemesia longinaris* individuals ± standard deviation in each locality.

Locality	Average distance (%)	Standard deviation (±)
Macaé	1.42	0.24
Ubatuba	1.07	0.19
Santos	1.25	0.43
Cananéia	0.81	0.25
São Francisco do Sul	1.37	0.26
Rio Grande	1.08	0.19
Mar del Plata	0.88	0.21

Table 3. *Artemesia longinaris*: average distance (%) among localities (numbers on bottom) ± standard deviation (values on top).

Locality	1	2	3	4	5	6	7
1 Macaé		0.19	0.25	0.21	0.22	0.19	0.19
2 Ubatuba	1.21		0.23	0.18	0.19	0.18	0.17
3 Santos	1.30	1.08		0.23	0.23	0.23	0.23
4 Cananéia	1.17	0.95	0.78		0.20	0.18	0.19
5 São Francisco do Sul	1.37	1.21	1.13	1.02		0.19	0.20
6 Rio Grande	1.20	1.04	1.13	0.96	1.20		0.17
7 Mar del Plata	1.16	0.96	0.97	0.83	1.11	0.95	

Table 4. Number of *Artemesia longinaris* individuals sampled, number of haplotypes, D.H. = haplotype diversity, and D.N. ± D.P. = nucleotide diversity ± standard deviation for each locality.

Locality	Number of samples	Number of haplotypes	D. H.	D. N. ± D. P.
Macaé	10	10	0.10	$1.38\times10^{-3} \pm 0.79\times10^{-3}$
Ubatuba	13	13	0.08	$1.05\times10^{-3} \pm 0.4\times10^{-3}$
Santos	2	2		
Cananéia	5	5	0.20	$0.80\times10^{-3} \pm 0.5\times10^{-3}$
São Francisco do Sul	9	9	0.11	$1.34\times10^{-3} \pm 0.8\times10^{-3}$
Rio Grande	10	9	0.12	$1.05\times10^{-3} \pm 0.6\times10^{-3}$
Mar del Plata	11	11	0.91	$0.87\times10^{-3} \pm 0.5\times10^{-3}$

es were obtained from this site. The caught of *Artemesia longinaris* in this locality is difficult, occurring only in some occasions with low temperatures and often in low abundances (Carvalho-Batista et al. 2011). The haplotype network did not reveal any genetic structure among groups (Fig. 4). Five haplotypes were shared, and the most frequent one was observed in four specimens from three localities (Fig. 4).

The analysis of molecular variance (AMOVA) did not detect structure among the localities, and the observed variation occurred predominantly within the localities. The FST indices were not significant ($p > 0.05$) (Table 5).

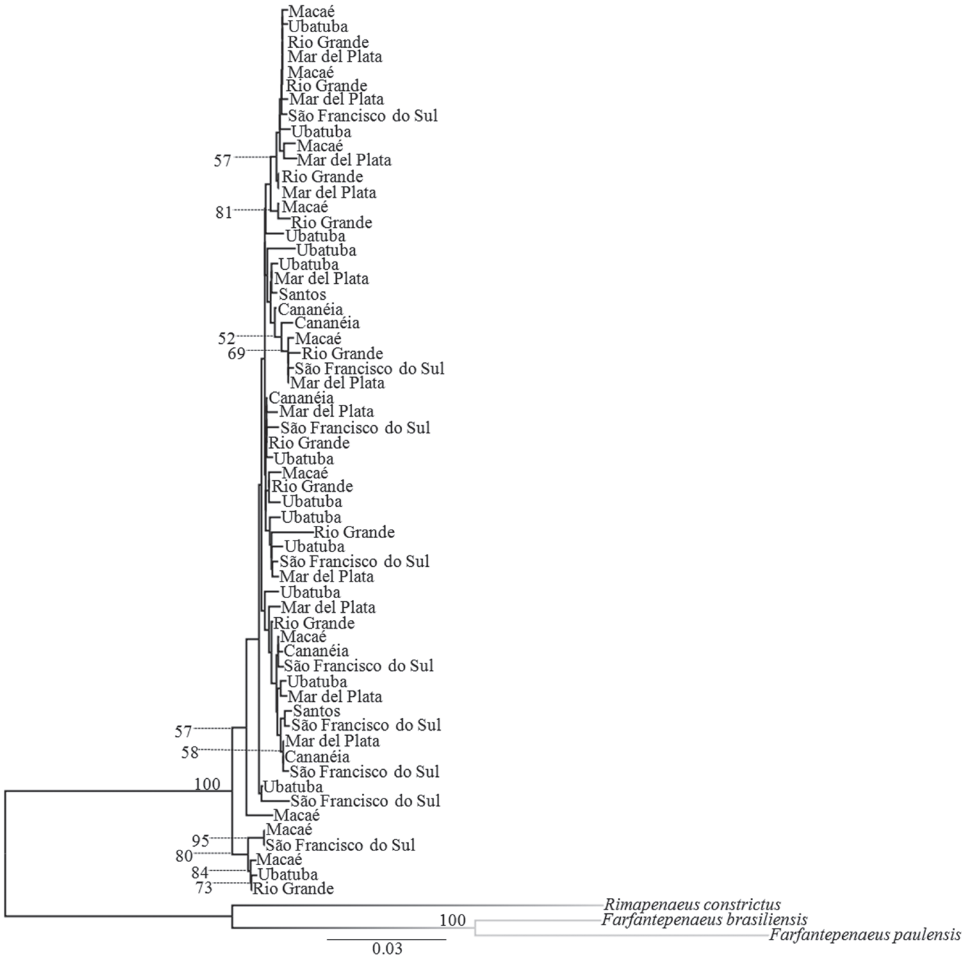

Figure 2. Dendrogram based on Neighbor-Joining distance method of COI gene sequences of individuals of *Artemesia longinaris*. Localities represent the analyzed specimens. Numbers are bootstrap support values (1000 replicates); values below 50% are not shown.

Discussion

The intraspecific genetic distance for *A. longinaris* (0–2.7%) is much lower than the interspecific distance between *A. longinaris* and the out-group species (21.3–27.1%). This result not only confirms *A. longinaris* as a single taxon throughout its distribution, but also supports the utilization of this methodology in the identification of penaeid shrimps from the Brazilian coast. The difference between the intra and interspecific genetic variation of the barcode region of the COI gene is termed the “barcode gap” (Hebert et al. 2004). It is an efficient method for differentiating species through the DNA Barcoding technique (Hebert et al. 2004, Waugh 2007, Frézal and Leblois 2008, Ward 2009). The genetic divergence values are consistent with other studies involving

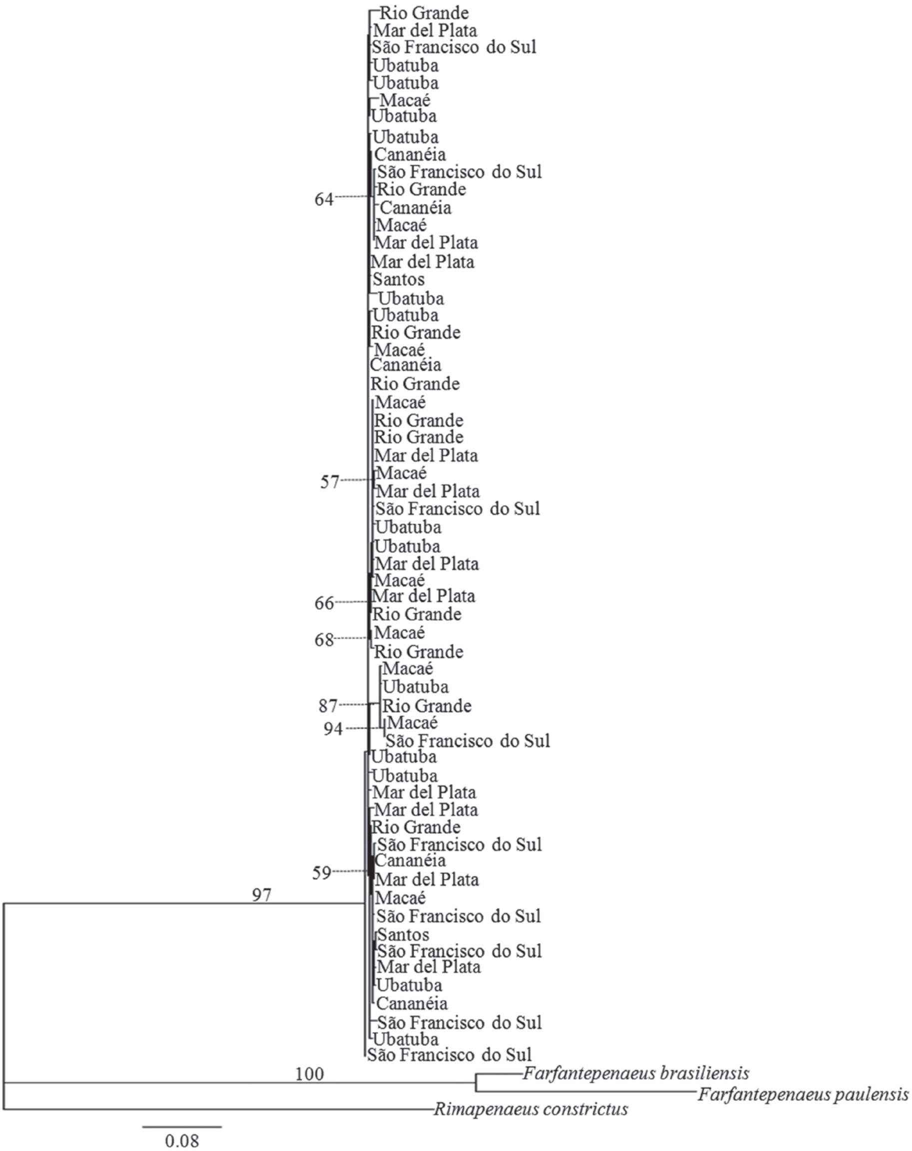

Figure 3. Phylogram for individuals of *Artemesia longinaris* inferred from Maximum Likelihood analysis of COI gene sequences. Localities represent analyzed specimens. Numbers are bootstrap support values (1000 replicates); values below 50% are not shown.

Table 5. Analysis of molecular variance (AMOVA) performed with specimens of *Artemesia longinaris* obtained from seven localities. *Significant values, $P < 0.05$.

Structure	Variation Source	%	Fixation index	P
Absent	Among localities	-1.80	FST: -0.02	0.95
	Within localities	101.80		

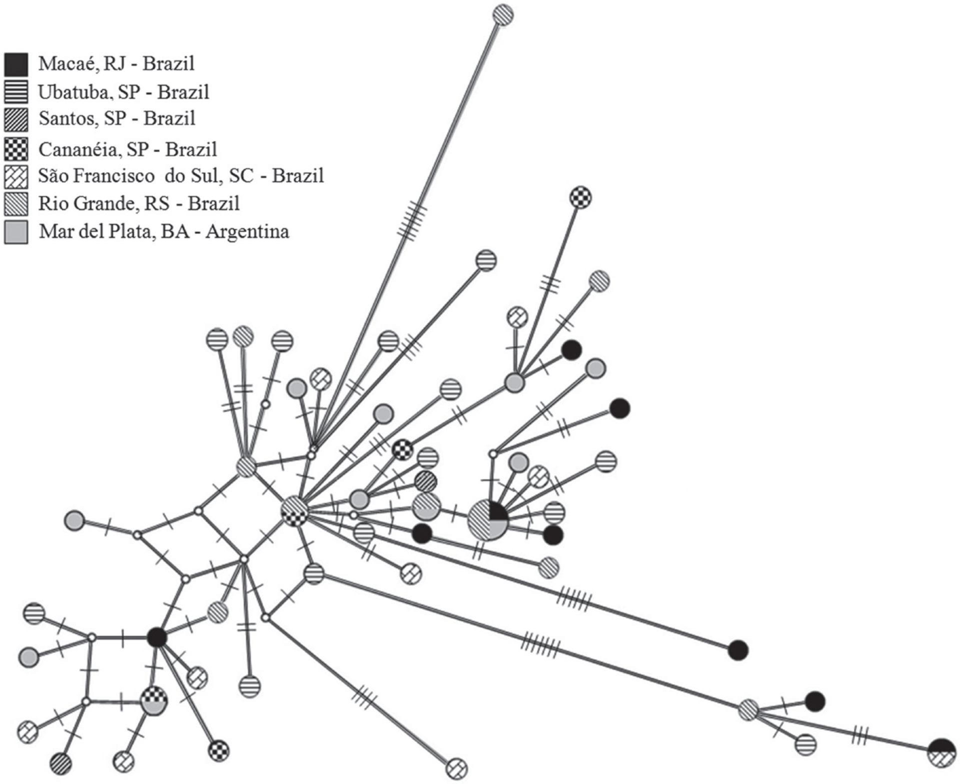

Figure 4. Haplotype network of *Artemesia longinaris* according to Median-Joining analysis. Each circle represent one haplotype found in the localities (53 haplotypes in 60 specimens). The size of the circle of each haplotype is proportional to its frequency in the sample. Each small dash represents a mutational step.

the family Penaeidae, with intraspecific values lower than 3.5% and interspecific values generally higher than 10% (in some cases exceeding 20%) (Gao et al. 2003, Quan et al. 2004, Keskin and Atar 2013).

Our analyses showed genetic homogeneity among the populations of *A. longinaris* along its entire geographical distribution. The FST value obtained reflects this absence of geographical genetic structure. In species with high genetic variation and few shared haplotypes, negative FST values are probably associated with the imprecision of the algorithms used in this type of analysis, and can be interpreted as zero (Winkelmann et al. 2013).

Despite the absence of significant genetic variability at the intraspecific level described here, phenotypic variability was previously observed among the populations of *A. longinaris* (see introduction). The determination of an individual phenotype is a consequence of the interaction between genotype and environment (Templeton 2006). Thus, the same genotype may be associated with different phenotypes under different environmental conditions (Miner et al. 2005, Vogt et al. 2008, Sotka 2012).

Recent studies with other decapods, with sampling at several points of the South American coast, found similar results on genetic homogeneity (Laurenzano et al. 2012, Terossi and Mantelatto 2012, Rossi and Mantelatto 2013, Wieman et al. 2013, Laurenzano et al. 2013). These authors indicated the high capacity of planktonic larval dispersal as the main factor responsible for this homogeneity over their distributions, making it impossible to establish a population structure over this broad geographical range (Gopurenko and Hughes 2002).

We can conjecture that similar larval dispersal occurs with *A. longinaris*, in which its larval development lasts 24 to 32 days, according to the temperature (Boschi and Scelzo 1977). This period is sufficient for the larvae to be passively transported for hundreds of kilometers by the currents (Palumbi 2003). The ability of larvae to travel for long distances was demonstrated for other penaeid shrimps. For example, larvae of *Pleoticus muelleri* (Spence Bate), on the Argentine coast, are able to travel for distances between 120 and 300 nautical miles (about 220 and 550 km, respectively), transported by the coastal currents (Boschi 1989).

It is thought that the dynamics of water masses in the region provides ideal conditions for larval drift of *A. longinaris* through the southwestern Atlantic. Coastal Water (CW), for instance, is a water mass that cover the geographical range of this study (Campos et al. 2000), and can flow towards north or south depending on the wind conditions and season showing different properties of temperature and salinity, depending the region and the influence of other water masses (Piola et al. 2005, Calado et al. 2006, Castro-Filho et al. 2008) allowing larval dispersal to different areas.

According to Fransozo et al. (2004), Costa et al. (2005) and Carvalho-Batista et al. (2011), the occurrence of adults of *A. longinaris* in São Paulo State is associated with the temperature decrease to 17–21 °C. During the spring (October to December) in Ubatuba, the number of animals in the larger size classes increased. It was associated with the coming of migrants into the population (Castilho et al. 2007a). Thus, the gene flow of *A. longinaris* is not limited to larval drift, but also is a consequence of juvenile and adult migration. Penaeid migration over long distances was also evidenced by Ruello (1975), who recaptured a female of *Melicertus plebejus* (Hess), on the Australian coast, 930 km from the site where the specimen was marked.

Our results, encompassing samples from its entire distribution, support the hypothesis that *A. longinaris* migrates over long distances, and is able to establish populations in different areas when conditions are appropriate. It is therefore possible to consider *A. longinaris* as a metapopulation, which fits the model of source and sink proposed by Pulliam (1988). The populations (or subpopulations) that are continuously more stable and in high density throughout the year, such as those from southern Brazil to Argentina and from Macaé (Boschi 1969ab, Nascimento 1983, Sancinetti 2011), are probably sources of new individuals for the less-stable populations, the sinks, such as the populations (or subpopulations) from São Paulo State.

Thus, these localities, where the populations are considered sources, would be strategic for the implementation of management measures such as the creation of protected areas or offseason periods, in order to maintain the fisheries in these areas and also in all range of its distribution. The role of marine protected areas in enhance fisheries in adjacent regions depend if they act as sources or as sinks (King 1995). Even connected one to each other, each subpopulation has its own dynamic (Begon et al. 2006), so the conservation policies must take into account the particular characteristics of each locality.

Studies investigating the larval dispersal and the migration of juveniles and adults of this species must be conducted in order to verify whether the model described by Pulliam (1988) is applicable or not. Apart from this, with the intent of providing a better quantification of the degree of exchange among the populations, as well as to evaluate the possibility of recent divergence among them, which is not detectable by the marker used here, additional molecular investigations using different genes are encouraged.

Conclusion

Our results confirm that the DNA barcoding technique is an efficient tool for the identification of penaeid shrimps from the Brazilian coast. In addition to the validation of *A. longinaris* as a single taxon, with no genetic differentiation among the populations through its entire geographical distribution, we showed the importance of the effect of the environmental conditions specific to each locality in the expression of the phenotypic characteristics of the individuals in a population.

The genetic homogeneity is maintained by the larval dispersal and high migratory capacity, which assure gene flow among populations. These characteristics make it possible for individuals to be transported by water masses and currents of the southwestern Atlantic.

In addition, this study also indicate the importance of populations of south Brazil and Macaé as sources, to provide individuals to other areas. Thus these populations should be considered essential in developing management strategies for the species.

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Differences in life-history and ecological traits between co-occurring *Panulirus* spiny lobsters (Decapoda, Palinuridae)

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Abstract

Coexistence of closely related species may be promoted by niche differentiation or result from interspecific trade-offs in life history and ecological traits that influence relative fitness differences and contribute to competitive inequalities. Although insufficient to prove coexistence, trait comparisons provide a first step to identify functional differences between co-occurring congeneric species in relation to mechanisms of coexistence. Here, a comparative review on life history and ecological traits is presented for two pairs of co-occurring species of spiny lobsters in the genus *Panulirus*: *P. gracilis* and *P. inflatus* from the Eastern Central Pacific region, and *P. argus* and *P. guttatus* from the Caribbean region. *Panulirus gracilis* and *P. inflatus* have similar larval, postlarval, and adult sizes and a similar diet, but differ in degree of habitat specialization, fecundity, and growth rate. However, little is known on behavioral traits of these two species that may influence their competitive abilities and susceptibility to predators. The more abundant information on *P. argus* and *P. guttatus* shows that these two species differ more broadly in degree of habitat specialization, larval, postlarval and adult sizes, diet, fecundity, growth rate, degree of sociality, defense mechanisms, susceptibility to predators, and chemical ecology, suggesting a greater degree of niche differentiation between *P. argus* and *P. guttatus* than between *P. gracilis* and *P. inflatus*. Whether the substantial niche differentiation and apparent interspecific trade-offs between *P. argus* and *P. guttatus* relative to *P. gracilis* and *P. inflatus* reflect an earlier divergence of the former pair of species in the evolution of the genus constitutes an intriguing hypothesis. However, whether or not post-divergence evolution of each species pair occurred in sympatry remains uncertain.

Keywords

Crustacea, Decapoda, Achelata, Palinuridae, coexistence, resource use, predation, Eastern Central Pacific, Caribbean region

Introduction

Spiny lobsters (Decapoda: Achelata: Palinuridae) are large (body length up to 60 cm), long-lived (> 10 years) crustaceans that occur in a wide range of habitats and depths, and constitute some of the most important fishing resources in all parts of the world (Phillips 2006). Spiny lobsters exhibit complex behaviors and are an important component of community structure and function because they consume a vast array of small benthic organisms and are prey to numerous species of higher predators (Lipcius and Eggleston 2000, Phillips et al. 2013). In addition, spiny lobsters are sturdy and easy to keep in controlled laboratory conditions, making them useful subjects for many types of biological, physiological, and behavioral studies (Cobb 2006).

The family Palinuridae comprises 54 extant species/subspecies arranged in 12 genera (Chan 2010), all of which have a specialized, flat-bodied larva called phyllosoma with multiple stages and a long (4–22 months) planktonic life, as well as a swimming, non-feeding postlarva called puerulus. The most diverse genus is *Panulirus*, with 24 species/subspecies, followed by *Jasus* and *Palinurus*, with six species each. These three genera contain the great majority of commercially important species. However, *Jasus* species are distributed exclusively in cold waters of the southern hemisphere (Jeffs et al. 2013), whereas *Palinurus* species are restricted to south east Africa and the north-eastern Atlantic, and generally occur at depths greater than 100 m (Groeneveld et al. 2013). In contrast, *Panulirus* species occur in shallow tropical and subtropical waters (< 100 m in depth) of both hemispheres, where the diversity of habitats may have promoted a greater radiation of this genus (George and Main 1967, George 2006). Therefore, the occurrence of two or more *Panulirus* species living in sympatry is common in different regions throughout the world (Briones-Fourzán and Lozano-Álvarez 2013).

The co-occurrence of congeneric species at local scales is common in many marine systems (e.g. Azovsky 1996, Sfenthourakis et al. 2005, González et al. 2011), but co-occurrence does not necessarily imply coexistence. The key criterion for coexistence is the "invasibility" criterion, which requires that each species must be able to increase from low density (i.e. persist) when the others are at their typical abundances (Chesson 2000). Conditions that are necessary but not sufficient for invasibility include negative density dependence and trade-offs in performance that influence population regulation (Siepielski and McPeek 2010). Trade-offs imply that advantages that one species may have over others are offset by compensating disadvantages (Chesson 2000, Kneitel and Chase 2004). For example, coexistence of species may be promoted by trade-offs between competitive ability and dispersal ability, between abilities to compete for alternative resources, between competitive ability and disturbance tolerance, and between competitive ability and susceptibility to predation or disease (Bohannan et al. 2002, Amarasekare 2003, 2008, Kneitel and Perrault 2006). These trade-offs may involve niche differentiation between species (McPeek 1996) or may result from interspecific trade-offs in life history and ecological traits that influence relative fitness differences and contribute to competitive inequalities (e.g. body size, fecundity, longevity, dispersal) (Tilman 1994, Chesson 2000, Amarasekare 2003, Amarasekare et al. 2004).

Because of the wealth of data needed, it is difficult to prove whether co-occurring species truly coexist, particularly for long-lived species wherein the relevant data should have to span multiple generations of each species (Siepielski and McPeek 2010, HilleRisLambers et al. 2012, Narwani et al. 2013). On the other hand, many studies related to coexistence have addressed species belonging to different genera, underscoring the need for more studies focusing on trying to understand the degree to which congeneric species that are within the same trophic level coexist, in particular at local scales (Siepielski and McPeek 2010). As species within genera are often predicted to be more similar to each other than between genera, trait-based approaches may provide a first step to identify functional differences between co-occurring congeneric species in relation to mechanisms of coexistence (Tilman 1994). Therefore, the aim of the present review is to compare life history and ecological traits between some coexisting *Panulirus* species as a first step to suggesting potential trade-offs that may promote their coexistence.

There are numerous studies addressing biological and/or ecological traits of spiny lobsters but few studies comparing traits between co-occurring species. For example, the co-occurrence of multiple *Panulirus* species in tropical waters of the Indo-West Pacific has been related to a differential use of habitats of adult lobsters across environmental gradients such as depth, turbidity, coral cover, and wave action (de Bruin 1969, Berry 1971, George 1974, Pitcher 1993, Coutures and Chauvet 2003), but there is little information on the life-history traits of these particular species. Therefore, emphasis is made in this review on two pairs of co-occurring congeneric species for which relatively more information is available, one from the Eastern Central Pacific region (*Panulirus gracilis* Streets, 1871 and *P. inflatus* (Bouvier, 1895)) and the other from the Caribbean region (*P. argus* (Latreille, 1804) and *P. guttatus* (Latreille, 1804)) (Fig. 1). Throughout the text, measurements are given as mean ± 95% confidence interval unless otherwise stated.

Eastern Central Pacific: *Panulirus gracilis* and *P. inflatus*

Panulirus gracilis occurs along the continental coast and islands from Peru to Mexico, and co-occurs with *P. inflatus* along most of the Pacific coast of Mexico (Holthuis 1991) (Fig. 1). These two species are so similar that they were considered as synonyms until Holthuis and Villalobos (1961) established that they constituted separate species. During 1976–1980, the biology, ecology and fisheries of both species were concurrently studied in Zihuatanejo, Mexico, by researchers from the National

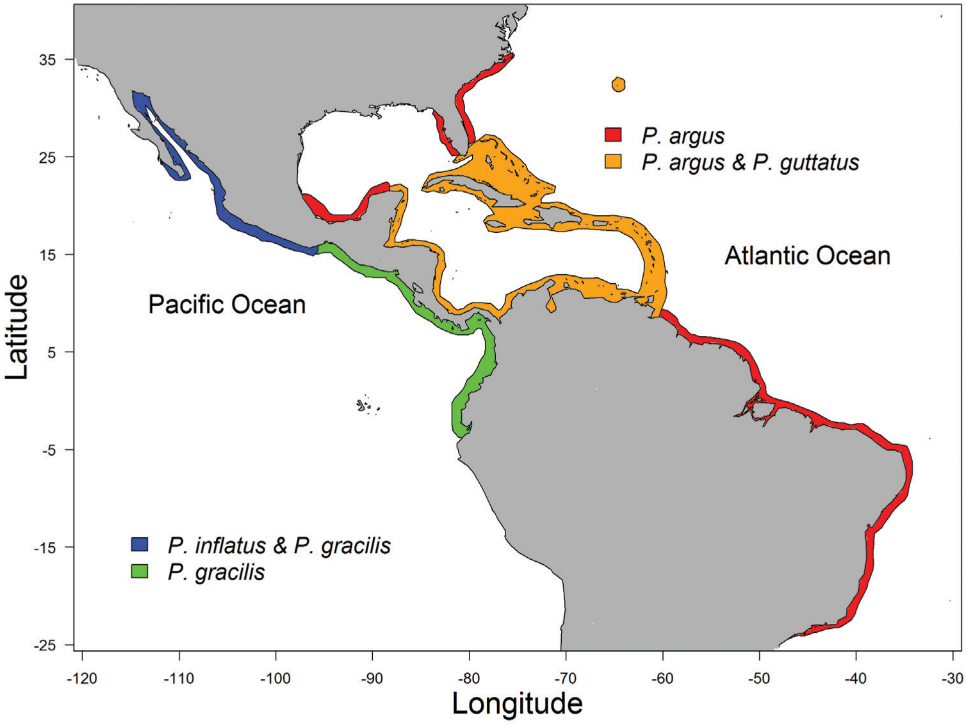

Figure 1. Geographic distribution of the two pairs of sympatric *Panulirus* species addressed in the text.

Autonomous University of Mexico. Comparative analyses of the original data from these and other studies provide insight into some differences in life history and ecological traits between these two species.

Larval and postlarval traits

Both *P. inflatus* (see Johnson and Knight 1966) and *P. gracilis* (see Báez 1983) have eleven phyllosoma stages which, according to Johnson (1971), are almost identical in almost every respect, except for the presence of a subexopodal spine on the fourth pereopod in stages five through eleven of *P. gracilis* and the absence of this spine in all stages of *P. inflatus*. However, molecular analyses have shown that this and other morphological criteria are insufficient to distinguish between phyllosomata of the two species (García-Rodríguez et al. 2008). The length of the larval phase has not been determined for either species.

Johnson (1971) described the puerulus of "*P. inflatus-gracilis*" from plankton samples collected over an area where the two species co-occur, whereas Báez (1983) described the puerulus of *P. gracilis* from samples collected in an area where only this species occurs. Both pueruli are similar in size (7.0–8.9 mm carapace length, CL) and have long, spatulated antennae, which are 2.7 times the length of the body in *P. gracilis* (see Báez 1983) and about 2 times the length of the body in "*P. inflatus-gracilis*" (see Johnson 1971). Based on these and other minor differences, Báez (1983) suggested that the puerulus of "*P. inflatus-gracilis*" described by Johnson (1971) belonged to *P. inflatus*.

Life history strategy and benthic distribution

Panulirus gracilis occupies different types of benthic habitats, from rocky bottoms with clear water to gravel-sand bottoms near river discharges where water can be considerably turbid, whereas *P. inflatus* occurs exclusively in rocky habitats with clear waters (Briones et al. 1981, Pérez-González et al. 1992, Pérez-González 2011). Although these findings suggest that *P. inflatus* might be considered a habitat specialist and *P. gracilis* a habitat generalist, an important criterion to consider a benthic species as habitat specialist is that its postlarvae settle into the same type of habitat where the adults live, and as habitat generalist that the postlarvae are able to settle in various types of habitats (Adams et al. 2006, Adams and Ebersole 2009); however, the natural settlement habitats have not been determined for either *P. inflatus* or *P. gracilis*. However, recently settled pueruli and early benthic juveniles of both species (6-24 mm carapace length, CL) were consistently found co-occurring among the profuse biota fouling the pylons of concrete piers in Zihuatanejo, but those of *P. inflatus* persist longer in this particular habitat, which resembles the rocky habitats occupied by *P. inflatus* adults (Briones-Fourzán and Lozano-Álvarez 2013). Also, Gracia and Lozano (1980) found numerous pueruli in the stomachs of benthic catfish *Occidentarius platypogon* (Günther, 1864) (previously known as *Netuma platypogon*), which they assigned to "*P. inflatus-gracilis*" following Johnson (1971). However, it is possible that those pueruli belonged to *P. gracilis* which, unlike *P. inflatus*, dwells in the same benthic habitats as *O. platypogon* (gravel-sand and muddy bottoms).

Body size, growth rate, and fecundity

Adults of *P. inflatus* and *P. gracilis* reach a similar body size (Fig. 2A). In Zihuatanejo, *P. inflatus* has a slightly larger mean size (CL) than *P. gracilis* (Fig. 2B), but mark-recapture data showed that *P. gracilis* grows almost twice as fast as *P. inflatus* (e.g. growth rate for males, *P. gracilis*: 0.96 ± 0.08 mm CL week^{-1}, *P. inflatus*: 0.56 ± 0.10 mm CL week^{-1}, Briones-Fourzán and Lozano-Álvarez 2003) (Fig. 2C). In this same location, the size of the smallest ovigerous females ever recorded and the size at which 50% of females are ovigerous (CL_{50}) were slightly larger for *P. gracilis* (47.5 mm CL and 80.0 mm CL, respectively) than for *P. inflatus* (45.6 mm CL and 77.5 mm CL, respectively) (Weinborn 1977, Gracia 1985, Briones-Fourzán and Lozano-Álvarez 1992). Both species have an extended yearly reproductive period during which individual females can produce up to four clutches (and possible more), with embryo development taking approximately three to four weeks before hatching (Briones-Fourzán and

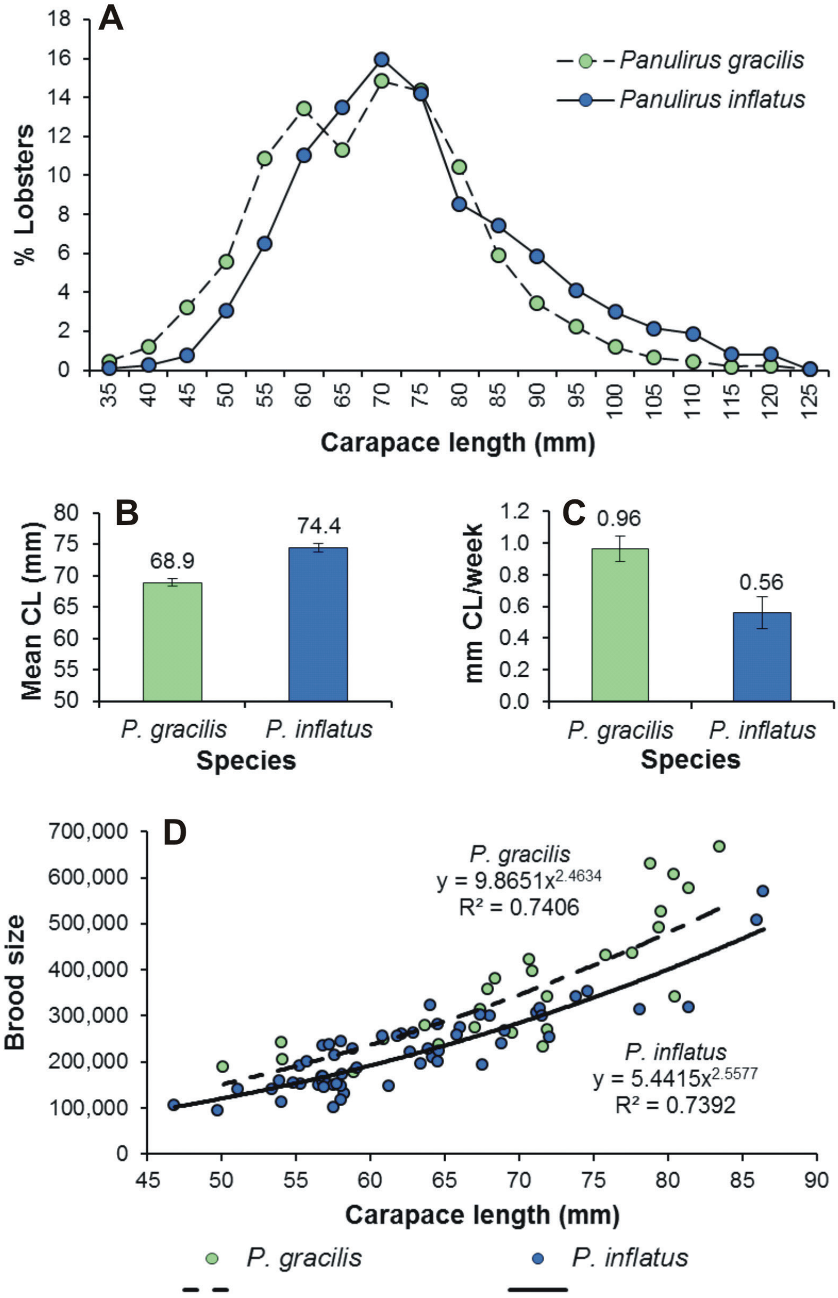

Figure 2. Differences in some life-history traits between *Panulirus gracilis* and *P. inflatus* from Zihuatanejo, Mexico. **A** carapace length (CL) distribution (n *P. gracilis*: 2162, n *P. inflatus*: 1873) **B** mean size **C** growth rate of males (mm CL week^{-1}, n *P. gracilis*: 148, n *P. inflatus*: 34) **D** brood size (number of eggs per clutch) versus CL relationship. Error bars denote 95% confidence intervals. (Data from **A, B** Briones-Fourzán and Lozano-Álvarez 1992,**C** Briones-Fourzán and Lozano-Álvarez 2003, **D** Gracia 1985, Fernández-Lomelín 1992).

Lozano-Álvarez 1992, Torres-Zepeda et al. 2008). Using original data on clutch size (number of eggs) versus CL concurrently taken for *P. inflatus* (see Gracia 1985) and *P. gracilis* (see Fernández-Lomelín 1992) from Zihuatanejo (Fig. 2D), an analysis of covariance (ANCOVA) showed that, after controlling for the significant effect of CL ($F_{1,82} = 231.71$, $p< 0.0001$), size-specific fecundity is significantly greater in *P. gracilis* than in *P. inflatus* ($F_{1,82} = 16.24$, $p< 0.001$) (Fig. 2D). Large broods are achieved partly through selection for smaller egg size. Pollock (1997) showed that, for spiny lobsters and other crustaceans, the number of eggs per gram of body weight provides an inverse index of egg size (i.e., the larger the index the smaller the egg). The use of this index shows that the eggs of *P. gracilis* (1047 ± 87 eggs g^{-1} body weight) are indeed significantly smaller than those of *P. inflatus* (911 ± 54 eggs g^{-1} body weight) (Student's *t*-test, $t_{85} = 2.685$, $p = 0.009$).

Use of habitat resources

Stomach content analyses showed that *P. gracilis* and *P. inflatus* consume various types of invertebrate prey but that both species exhibit a marked preference for molluscs (Lozano-Álvarez and Aramoni-Serrano 1996) (Fig. 3). In Zihuatanejo, a capture-recapture experiment was conducted during 1979–80 to estimate monthly lobster densities on a 36-ha rocky site ("site A") where the two species co-occurred (Lozano et al. 1982). At the same time, the seasonal composition of the benthic community at site A and other sites, as well as the seasonal changes in condition factor of the two lobster species were studied (Aramoni-Serrano 1982, Lozano-Álvarez and Aramoni-Serrano 1996). The total density of lobsters on site A showed a marked increase in September-October relative to the other months (Fig. 4A). For each separate species, the density showed values ≤ 15 ind. ha^{-1} between April and August, but then more than doubled in September. In October, the density of *P. inflatus* doubled again while that of *P. gracilis* decreased to previous levels. By November, the density of *P. inflatus* also decreased to previous levels (Fig. 4A).

Interestingly, site A (but not other sites) exhibited a peak in relative abundance of molluscs in the autumn (Fig. 4B), suggesting that this particular site became a food-rich habitat patch for lobsters during this season (Aramoni-Serrano 1982) and potentially explaining the substantial increase in local density of both *P. inflatus* and *P. gracilis* in September, which possibly reached the carrying capacity of the site. In October, the increase in density of *P. inflatus* and decrease in density of *P. gracilis* was followed by the recapture of several *P. gracilis* lobsters during October to December on a gravel-sand site 3 km away from site A (Lozano et al. 1982), suggesting that *P. inflatus* was able to displace *P. gracilis* from the food-rich patch. However, the high density of *P. inflatus* during the autumn possibly resulted in intraspecific competition for food resources, further exacerbated by the local decrease in abundance of molluscs during the winter (Lozano-Álvarez and Aramoni-Serrano 1996), as indicated by the significantly lower condition factor of *P. inflatus* during the winter relative

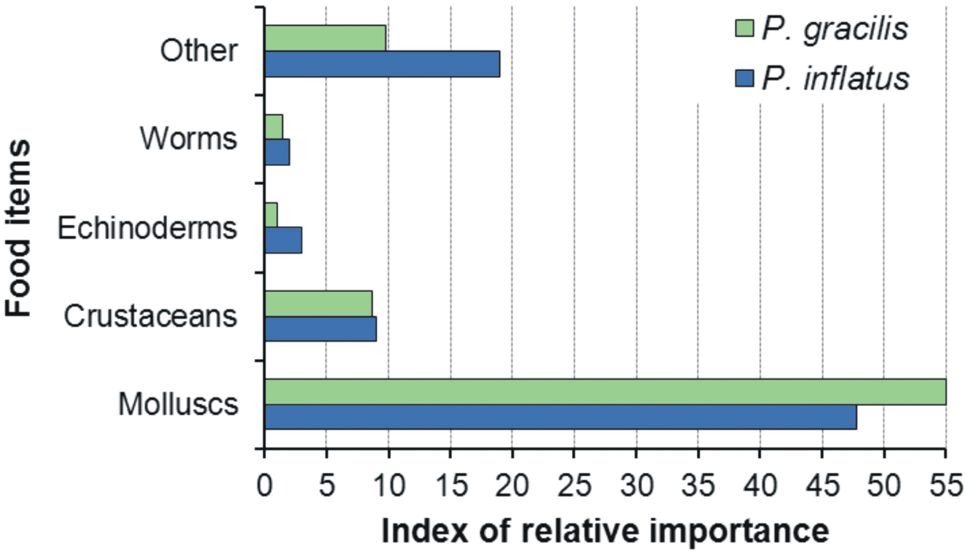

Figure 3. Diet of *Panulirus gracilis* and *P. inflatus* from Zihuatanejo, Mexico. For each food item the index of relative importance (IRI) is estimated as IRI = (% frequency × % weight)/100. (Data from Lozano-Álvarez and Aramoni-Serrano 1996).

to other seasons and to *P. gracilis* (Fig. 4C). In conjunction, these findings suggest that *P. inflatus* is the superior competitor in the rocky habitats to which this species is restricted.

Greater Caribbean region: *Panulirus argus* and *P. guttatus*

Panulirus argus and *P. guttatus* co-occur throughout the Greater Caribbean region (see Fig. 1). Some of the following sections are based on studies on these lobsters conducted by researchers from the National Autonomous University of Mexico in the Caribbean coast of Mexico, where life history traits and ecological aspects of these species have been concurrently studied for over 20 years.

Larval and postlarval traits

Lewis (1951) described eleven phyllosoma stages for *P. argus* from plankton samples and estimated the larval duration in about six months, but Goldstein et al. (2008), who obtained the complete larval phase in the laboratory, identified only ten distinct stages with the entire larval duration in these controlled conditions varying between 4.6 and 6.6 months. The early phyllosoma stages of *P. guttatus* have not been described and the larval duration of this species has not been determined. According to Chitty (1973),

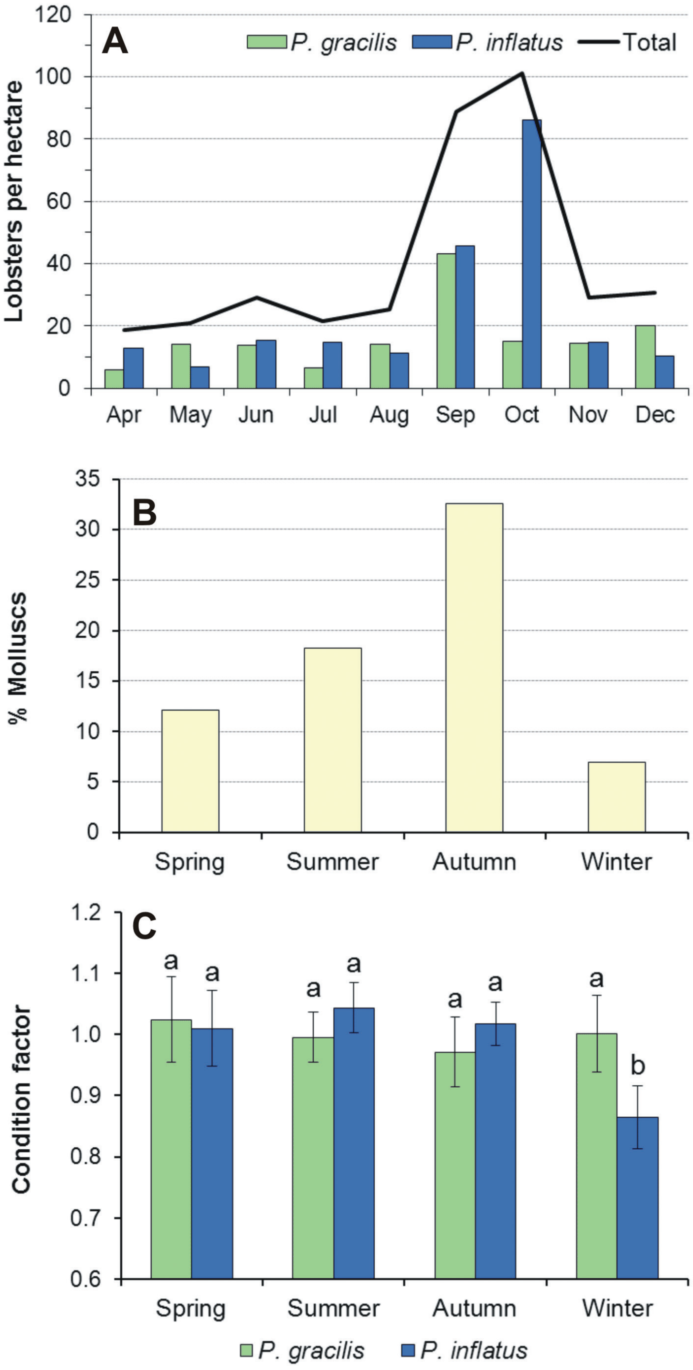

Figure 4. Potential ecological interactions between *Panulirus gracilis* and *P. inflatus* in a rocky site ("Site A") in Zihuatanejo, Mexico. **A** lobster density (number of individuals ha^{-1}) **B** relative abundance of molluscs (percentage of molluscs in benthic samples) **C** condition factor of lobsters. Error bars denote 95% CI. (Data from **A** Lozano et al. 1982, **B** Aramoni-Serrano 1982, **C** Lozano-Álvarez and Aramoni-Serrano 1996).

first-stage phyllosomata of *P. argus* and *P. guttatus* are virtually indistinguishable based on morphology and size. However, stages VI to X of *P. guttatus* are substantially larger than the corresponding stages of *P. argus* (Baisre and Alfonso 1994).

The puerulus of *P. argus* is relatively small (6.1 mm CL on average) and has tapered antennae about 1.5 times the length of the body (Lewis et al. 1952, Goldstein et al. 2008). In contrast, the puerulus of *P. guttatus* is quite large (10 mm CL) and has long, spatulated antennae about 2.5 times the length of the body (Briones-Fourzán and McWilliam 1997). However, upon molting into the first juvenile stage and as individuals continue to grow, the antennae of *P. guttatus* become progressively shorter and thinner than those of *P. argus* (Briones-Fourzán et al. 2006).

Life history strategy and benthic distribution

It is well known that *P. argus* is an ontogenetic shifter, i.e. a species wherein the postlarvae settle into habitats distinct from those of the adults and further undergo notable ontogenetic habitat shifts toward the adult habitat (Adams and Ebersole 2009). The pueruli of *P. argus* settle in vegetated habitats of shallow reef lagoons and bays (seagrass meadows, macroalgal beds, coastal mangroves), where the early benthic juveniles remain for a few months, taking shelter among the vegetation. Eventually, juveniles outgrow the protection afforded by the vegetation and seek shelter in any crevice-type structure in or adjacent to the settlement habitats before gradually moving to the coral reef habitats where the subadults and adults dwell (Butler et al. 2006). Mating and brooding of *P. argus* occur in the reef habitat, but after embryo development – which takes from three to four weeks – is completed, the females move to deeper areas to release the phyllosoma larvae (Bertelsen 2013), which develop in oceanic waters.

Upon changing habitats, ontogenetic shifters also tend to undergo changes in behavior (Adams et al. 2006). Indeed, after their first benthic habitat shift, *P. argus* lobsters change from being asocial to being highly gregarious, with multiple individuals commonly sharing individual crevice shelters (Childress and Herrnkind 1996). In addition, *P. argus* has a highly mobile lifestyle, with movement ranges increasing with lobster size. In some locations, these movements include organized mass migrations over tens to hundreds of kilometers (Herrnkind 1969).

By contrast, *P. guttatus* is a habitat specialist, as the pueruli of this species settle directly into the coral reef habitat where the juveniles and adults also dwell (Briones-Fourzán and McWilliam 1997). Individuals of *P. guttatus* are highly sedentary, with a home range for adults of approximately 100 m in radius (Lozano-Álvarez et al. 2002). Therefore, growth, mating, brooding, and egg hatching all take place in the coral reef habitat (Briones-Fourzán and Contreras-Ortiz 1999, Negrete-Soto et al. 2002). Individuals of *P. guttatus* use reef crevices as shelters, and although small groups can share crevices, many individuals dwell solitarily, reflecting a much lower degree of gregariousness than that exhibited by *P. argus* (Briones-Fourzán 1995, Sharp et al. 1997, Briones-Fourzán and Lozano-Álvarez 2008).

Body size, growth rate, and fecundity

Adults of *P. argus* and *P. guttatus* have a very different body size (Fig. 5A). For example, in the Puerto Morelos coral reef, *P. argus* has a much larger mean size (82.3 ± 2.24 mm CL) than *P. guttatus* (59.0 ± 0.83 mm CL) (Fig. 5B) and the former species also grows much faster than the latter (weekly growth rate for males, *P. argus*: 0.91 ± 0.6 mm CL week^{-1}, *P. guttatus*: 0.26 ± 0.13 mm CL week^{-1}) (Lozano-Álvarez et al. 1991, Negrete-Soto et al. 2002) (Fig. 5C). In the same location, the largest ovigerous female of *P. guttatus* ever recorded (73.5 mm CL) was smaller than the smallest ovigerous female of *P. argus* ever recorded (75.0 mm CL). In both species, large females can produce up to four broods per year (Cruz and de León 1991, Briones-Fourzán and Contreras-Ortiz 1999), but the CL_{50} of ovigerous females is 95.5 mm CL for *P. argus* and 59.0 mm CL for *P. guttatus* (Briones-Fourzán 1995). Due to the large interspecific difference in size, size-specific fecundity is far larger in *P. argus* than in *P. guttatus* (Fonseca-Larios and Briones-Fourzán 1998, Briones-Fourzán and Contreras-Ortiz 1999) (Fig. 5D), more so when the size of the eggs is taken into account. As Pollock (1997) had previously noted, the number of eggs per gram of body weight is significantly larger (indicating smaller eggs) in *P. argus* (689 ± 27) than in *P. guttatus* (519 ± 15) (t_{322} = 10.925, p < 0.0001).

Use of reef resources

The benthic distribution of *P. argus* and *P. guttatus* overlaps in the coral reef habitat. In Puerto Morelos, *P. guttatus* outnumbers *P. argus* by 5 to 1 across the entire reef habitat, but the relative density of each species varies with reef zone. Thus, the ratio of *P. guttatus* to *P. argus* is, on average, 2:1 in the back reef (the protected reef zone facing the mainland), but 16:1 in the fore reef (the exposed reef zone facing the open waters) (Lozano-Álvarez et al. 2007). A numerical dominance of *P. guttatus* over *P. argus* on fore reefs has also been reported in Florida (Sharp et al. 1997) and Belize (Acosta and Robertson 2003). However, there is no evidence that *P. guttatus* can displace *P. argus* via interference competition because individuals of these congeneric species do not act aggressively toward each other even when in close proximity (Lozano-Álvarez and Briones-Fourzán 2001). Rather, there is evidence that *P. guttatus* and *P. argus* make a differential use of reef resources (Lozano-Álvarez et al. 2007). For example, although lobsters of both species feed on a wide variety of organisms with a marked preference for crustaceans and molluscs (Colinas-Sánchez and Briones-Fourzán 1990) (Fig. 6), interspecific competition for food resources is unlikely, as individuals of *P. guttatus* forage on the reef itself (Wynne and Côté 2007) whereas reef-dwelling individuals of *P. argus* forage on seagrass and soft-bottom areas adjacent to the coral reefs (Cox et al. 1997, Briones-Fourzán et al. 2003).

Also, *P. argus* lobsters tend to occupy crevices ('dens') along the lower and middle portions of the reef and *P. guttatus* lobsters over the middle and upper portions of the reef (Lozano-Álvarez et al. 2007). Occupation of individual dens by multiple conspecifics is more common for *P. argus*, whereas occupation of individual dens by solitary

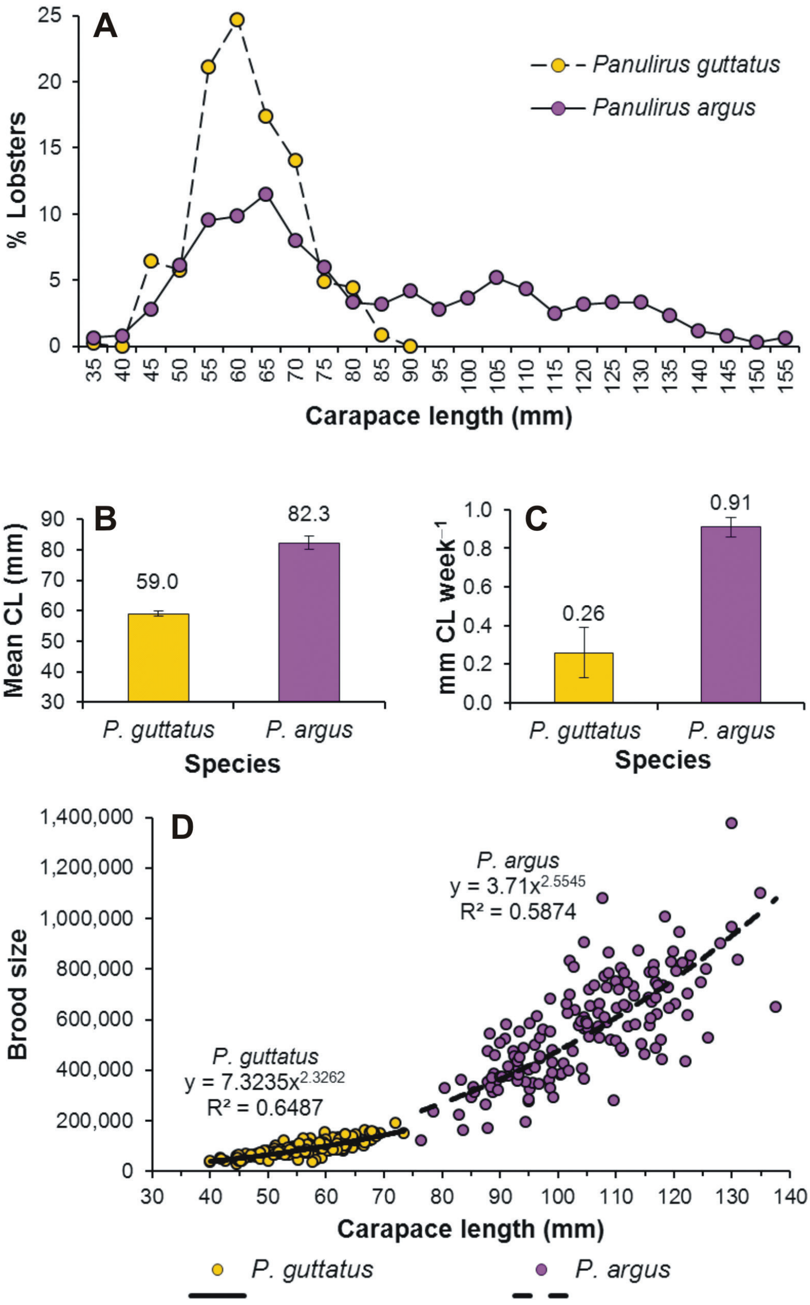

Figure 5. Differences in some life-history traits between *Panulirus argus* and *P. guttatus* from Puerto Morelos, Mexico. **A** carapace length (CL) distribution (n *P. argus*: 717, n *P. guttatus*: 450) **B** mean size **C** growth rate of males (mm CL week^{-1}, n *P. argus*: 148, n *P. guttatus*: 57) **D** brood size (number of eggs per clutch) versus CL relationship. Error bars denote 95% confidence intervals. (Data from **A, B** Lozano-Álvarez et al. 2007, Briones-Fourzán and Lozano-Álvarez 2013, **C** Negrete-Soto et al. 2002, **D** Fonseca-Larios and Briones-Fourzán 1998, Briones-Fourzán and Contreras-Ortiz 1999).

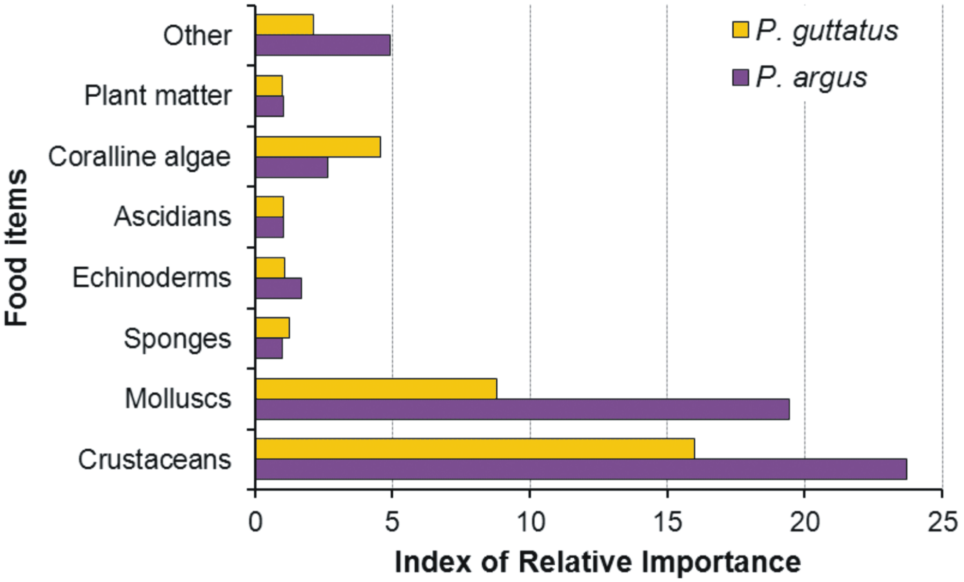

Figure 6. Diet of *Panulirus argus* and *P. guttatus* from Puerto Morelos, Mexico. For each food item the index of relative importance (IRI) is estimated as IRI = (% frequency × % weight)/100. (Data from Colinas-Sánchez and Briones-Fourzán 1990).

individuals is more common for *P. guttatus*. Moreover, in dens harboring either species separately or both species together, *P. argus* lobsters typically occupy the floor and the entrances of the dens, while *P. guttatus* lobster typically occupy the deep recesses of the dens, clinging to the ceiling or walls (Sharp et al. 1997, Lozano-Álvarez et al. 2007, Briones-Fourzán and Lozano-Álvarez 2008). The differential pattern of occupation of the den space by these congeners appears to minimize competition for shelter and to be related with a differential susceptibility to predators.

Susceptibility to predators

An important ecological trade-off that favors local coexistence of similar species within the same trophic level is a differential susceptibility to predators (Amarasekare 2008). This has been explored for *P. guttatus* and *P. argus*, which are potential prey to the same predators in the coral reef habitat (e.g. Randall 1967). In field studies, a negative relationship between the abundances of predators and prey would suggest that the abundance of predators controls the abundance of prey (e.g. Sih 1984, Hixon and Beets 1993, Eggleston et al. 1997). In the Puerto Morelos coral reef, Lozano-Álvarez et al. (2007) examined the relationship between the densities of predators and lobsters of each species by reef zone. A negative relationship emerged only for *P. guttatus* at the fore-reef zone, where this species was numerically dominant.

In the laboratory, individuals of *P. guttatus* and *P. argus* differed significantly in performance of several defense mechanisms expressed by spiny lobsters, indicating a differential defense strategy for each species (Briones-Fourzán et al. 2006). In particular, *P. argus* relies heavily on the formidable defense of its long, strong spiny antennae, especially when multiple individuals join in cooperative defense, whereas *P. guttatus*, which has much thinner and weaker antennae, does not express communal defensive behavior at all. *Panulirus guttatus* lobsters are also more cryptic and only emerge from their shelters to forage for short periods during the night. Individuals of *P. argus* have to traverse open areas to forage and tend to remain stationary to minimize detection when a predator approaches. If a predator attacks, a *P. argus* lobster can confront the predator, turning deftly to face it at all times, lashing and raking at the predator with its antennae. By contrast, if an individual of *P. guttatus* detects an approaching predator, it retreats backwards into the nearest available reef crevice, and if attacked, it can swim backwards in a slow but protracted bout that will effectively remove it from the visual field of the predator. In essence, *P. guttatus* exhibits a shy behavioral type and a higher susceptibility to predators, whereas *P. argus* exhibits a bold behavioral type and a lower susceptibility to predators (Briones-Fourzán et al. 2006).

Chemical ecology

The behavior of spiny lobsters is largely mediated by chemical communication (Aggio and Derby 2011). Because shelter is a limiting resource for these lobsters, individuals that are seeking shelter tend to be attracted to chemical scents released by sheltered conspecifics ("attraction cues"). On the other hand, avoiding scents from a lethally injured or freshly killed conspecific ("alarm odors"), which may signal the proximity of a predator, is a particularly effective antipredator strategy for gregarious species (Dicke and Grostal 2001). However, the degree of gregariousness varies widely among spiny lobsters (Childress 2007) and is particularly different between *P. argus* and *P. guttatus* (see Briones-Fourzán and Lozano-Álvarez 2008).

Briones-Fourzán et al. (2008) compared how individuals of *P. argus* and *P. guttatus* responded to attraction cues and alarm odors from either conspecifics or congeners. As expected, individuals of both species were significantly attracted to shelters emanating conspecific attraction cues but responded neutrally to shelters emanating congeneric attraction cues. However, individuals of *P. guttatus* responded neutrally to shelters emanating either conspecific or congeneric alarm odors, whereas individuals of *P. argus* significantly avoided shelters emanating either conspecific or congeneric alarm odors. The differential responses to alarm odors between species suggest that the cryptic defensive behavior of *P. guttatus* appears to be sufficiently adaptive to offset the need to avoid dens with conspecific (and congeneric) alarm odors, whereas learning to avoid dens with alarm odors from *P. guttatus* likely increases fitness in reef-dwelling *P. argus*, which leave their reef shelters to forage elsewhere during the night and then have to return to the reef to shelter during the day (Briones-Fourzán et al. 2008).

Discussion

The present study basically describes differences and similarities in traits between *Panulirus* species that co-occur both regionally and locally. Although just showing that species differ phenotypically or ecologically is insufficient to assign those differences to the type of trade-off necessary to promote coexistence (Siepielski and McPeek 2010), differences in morphological, physiological, ecological, and behavioral traits can help generate hypotheses regarding niche differentiation and interspecific trade-offs that influence relative fitness differences and contribute to competitive inequalities (e.g. body size, fecundity, longevity, dispersal) that may lead to coexistence, especially between congeneric species that co-occur at local scales (Tilman 1994, Amarasekare 2003, HilleRisLambers et al. 2012).

Panulirus argus and *P. guttatus* differ widely in their degree of habitat specialization and exhibit broad differences in many life history and ecological traits (e.g. larval and postlarval size and morphology, adult body size, fecundity, growth rate, movement range, behavior, susceptibility to predators) (Table 1). The large differences between *P. argus* and *P. guttatus* suggest the existence of important trade-offs leading to a stable coexistence of these two congeners. For example, although these congeners share the reef habitat, *P. guttatus* is better at exploiting shelter and food resources in this habitat, but is more susceptible to predators relative to *P. argus*. In contrast, *P. inflatus* and *P. gracilis* appear more similar in some traits (e.g. larval, postlarval, and adult size, diet) but they differ in other traits (e.g. fecundity, growth rate) and in habitat use, suggesting interspecific trade-offs that may contribute to competitive inequalities (Table 2). However, much remains to be investigated on the chemical ecology and behavioral traits of *P. gracilis* and *P. inflatus* that may influence their competitive abilities and susceptibility to predators (Table 2).

An intriguing hypothesis would be whether the substantial niche differentiation and apparent interspecific trade-offs between *P. argus* and *P. guttatus* relative to *P. gracilis* and *P. inflatus* reflect an earlier divergence of the former pair of species in the evolution of the genus. Several phylogenetic analyses (e.g. McWilliam 1995, Ptacek et al. 2001, Patek and Oakley 2003, George 2006) concur in that there are two major lineages in the radiation of *Panulirus*, with species in the first lineage representing an earlier radiation than species in the second lineage. The first lineage likely radiated from an '*argus*-like ancestor' from which *P. argus* split, possibly in the Mid-Miocene (18–8 mya) (Ptacek et al. 2001, George 2006). Morphological and molecular criteria place *P. guttatus* in the first lineage as well, but the origin of this species remains uncertain as the late phyllosmata and the puerulus of *P. guttatus* exhibit the long, spatulated antennae typical of species in the second lineage, probably as a result of early divergence (McWilliam 1995, Ptacek et al. 2001, George 2006). In contrast, *P. gracilis* and *P. inflatus* clearly belong to the second major lineage and these two species constitute a single clade, with *P. inflatus* possibly splitting from *P. gracilis* as recently as the late Miocene/Pliocene (8–2 mya) (George 2006).

However, differences between the two pairs of co-occurring species due to divergence times alone would imply that speciation occurred in ecological sympatry, and at

Table 1. Summary of differences in life-history and ecological traits between *Panulirus guttatus* and *P. argus* living in sympatry in the Caribbean region.

Life history or ecological trait	*Panulirus guttatus*	*Panulirus argus*
Life-history style	Habitat specialist	Ontogenetic shifter
Larval and postlarval size	Larger	Smaller
Adult size	Smaller	Larger
Growth rate	Slower	Faster
Brood size	Smaller	Larger
Egg size	Larger	Smaller
Diet	Similar?	Similar?
Foraging habitats	Coral reef	Seagrass, rubble areas
Lifestyle	Highly sedentary	Highly mobile
Degree of gregariousness	Lower	Higher
Behavioral type	Shy	Bold
Susceptibility to predators	Higher	Lower
Response to conspecific alarm odors	Neutral	Avoidance
Response to congeneric alarm odors	Neutral	Avoidance
Competitive rank (in reef habitat)	Superior	Inferior

Table 2. Summary of differences in life history and ecological traits between *Panulirus inflatus* and *P. gracilis* living in sympatry in the Eastern Central Pacific region.

Life-history or ecological trait	*Panulirus inflatus*	*Panulirus gracilis*
Life-history strategy	Habitat specialist?	Habitat generalist?
Larval and postlarval size	Similar	Similar
Adult size	Similar	Similar
Growth rate	Slower	Faster
Brood size	Smaller	Larger
Egg size	Larger	Smaller
Diet	Similar?	Similar?
Foraging habitats	Rocky areas	Rocky + gravel-sand areas
Lifestyle	Mobile	Highly mobile
Degree of gregariousness	?	?
Susceptibility to predators	?	?
Behavioral type	?	?
Response to conspecific alarm odors	?	?
Response to congeneric alarm odors	?	?
Competitive rank	Superior?	Inferior?

least some speciation in the genus *Panulirus* appears to have been the result of vicarious events associated with major changes in oceanic currents (affecting larval dispersion) due to continental plate movements (George 2006). For example, a recent range expansion northward by *P. gracilis* into the historical range of *P. inflatus* could mean sympatry between these two species is much more recent than their time of divergence.

Moreover, if life history traits such as growth rate, size at maturity, and fecundity tend to be more similar for species that occupy more similar microhabitats, this could partially explain the greater overlap between *P. gracilis* and *P. inflatus* than between *P. argus* and *P. guttatus*. For each pair of species, these hypotheses would have to be tested either via manipulative experiments involving removal or exclusion of one species to measure its impact on the other and vice versa, or by comparing locations where both species co-occur to locations where either species is absent.

More quantitative studies are also needed to determine how much overlap in the use of food resources truly exists between co-occurring species. Spiny lobsters are omnivorous consumers, but stomach content analyses suggest that some co-occurring species prefer different types of prey (e.g. Colinas-Sánchez and Briones-Fourzán 1990). The use of stable isotope analyses (SIA) (e.g. Waddington et al. 2008) may help to better define the trophic level of co-occurring *Panulirus* species. For example, a recent study using SIA in *P. guttatus* and *P. argus* from Puerto Morelos showed that small carnivores contribute more to the diet of adult *P. guttatus* whereas small herbivores contribute more to the diet of reef-dwelling *P. argus* (Segura-García et al. unpublished data). Similarly, high through-put DNA sequencing techniques (e.g. O'Rorke et al. 2012) may help identify a potential resource partitioning between the otherwise similar phyllosoma larvae of *P. gracilis* and *P. inflatus*.

Identifying mechanisms of coexistence for congeneric species that live in sympatry is an important issue for the establishment of marine protected areas by allowing identification of species that have broad or narrow habitat requirements (McPeek 1996, Acosta and Robertson 2003). It may also provide insight into how these species could respond to climate change and other human-mediated environmental impacts such as habitat loss, degradation, and fragmentation, as well as the introduction of invasive species (McPeek 1996, HilleRisLambers et al. 2012), all of which constitute pressing issues for the shallow-water *Panulirus* species (Briones-Fourzán and Lozano-Álvarez 2013).

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RESEARCH ARTICLE

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Reference values for feeding parameters of isopods (*Porcellio scaber*, Isopoda, Crustacea)

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Abstract

The advantage of using terrestrial isopods in toxicity studies is that a battery of parameters can be tested at different levels of biological complexity. Feeding parameters for example link organism level response to potential ecological consequences but a problem with using feeding parameters in toxicity tests with terrestrial isopods is their high variability. The aim of our study was to set benchmark values for feeding parameters for isopod *Porcellio scaber* (Isopoda, Crustacea) in laboratory-controlled experiments. In the work presented here, the daily feeding rate of the central 50% of the control population of *P. scaber* and a correlation between feeding rate and isopod weight were set. Values outside these ranges need additional evaluation to increase the relevance of test outcomes. We suggest using benchmark values for feeding parameters as well as the coefficient of variation (a) to identify animals with altered feeding parameters with respect to controls, and (b) to assess the data quality in each experiment.

Keywords

Feeding behaviour, toxicity testing, standard tests

Introduction

Numerous studies have analysed feeding behaviour and food preferences of terrestrial isopods, which have an important ecological role as decomposers of leaf litter and are one of the model organisms used in laboratory toxicity testing and terrestrial ecotoxicology (Drobne and Hopkin 1994, Drobne 1997, Loureiro et al. 2006, Zidar et al. 2012).

Terrestrial isopods are saprophagous animals and have no single food source that meets all their nutritional needs. Only a mixture of several food items is an optimal nourishment (Zimmer 2002). In terrestrial environments, there is a significant imbalance of nutrients and in addition, many potential foods contain unfavourable concentrations of deterrents or other toxic compounds. Consequently, terrestrial isopods have acquired different feeding strategies, which allow them to respond to spatial and temporal variations in the quality and quantity of food (for review: Zimmer 2002).

Adaptations in feeding behaviour have allowed terrestrial isopods to reduce food consumption or be selective among different food items (Zidar et al. 2012). Their compensatory ability when consuming “low-quality” food to lengthen the food retention time in the gut is important (Wieser 1984). Such feeding behaviour responses to different foods are used in toxicity tests as organism level responses, which can be used to assess the effects of chemicals added to food (Drobne and Hopkin 1994, Drobne 1997, Loureiro et al. 2006). In toxicity studies, rates of feeding and faecal production as well as food assimilation efficiency are calculated (Drobne and Hopkin 1994, Loureiro et al. 2006, Novak et al. 2013). Reduction in feeding over that of control animals at a certain concentration of chemicals is taken as a measure of the effect at a given exposure dose (Drobne and Hopkin 1994). A significant benefit of using feeding parameters as toxicity endpoints is their potential to anticipate population and ecosystem consequences (De Coen and Janssen 2003, Lardies et al. 2004).

Since food consumption is indicative of the overall condition of an organism (De Coen and Janssen 2003), it is used as an indirect measure of fitness of the organism. It has been proven that in herbivorous arthropods, life history traits often depend upon diet quality. Lardies et al. (2004) provided evidence that in the terrestrial isopod *Porcellio laevis* the quality of dietary components has an effect on growth rate, size and number of offspring and incubation period, trade-offs between reproductive investment, growth, and survival, as well as the growth rate of offspring. Rushton and Hassall (1983) additionally reported that food quality affects population dynamics in isopods, presenting evidence that the growth rate of juvenile *Armadilldium vulgare* increased exponentially with the increasing proportion of high-quality food in the diet.

An intrinsic characteristic of feeding parameters is their relatively high variation. This fact is interpreted as “noise” when a desired signal is measured. As a result, the power of isopod toxicity tests with feeding rate as a measured response is reduced, and quantification of the response (signal) to toxicants is of limited power. In toxicity tests an adverse effect is proven when the signal-to-noise ratio equals or exceeds a certain value and when a clear dose-effect response is observed (Van der Lelie et al. 1997).

The aim of our study was to set the limits for feeding parameters of the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) in laboratory-controlled experiments, which could serve as a benchmark for feeding rate of control, non-stressed animals. We analysed feeding parameters of about 600 animals in 60 experiments. In the analysed specimens, several sources of biological variability were retained to mimic to some degree the characteristics of isopod populations in nature. The sources of variability included: sex, moulting, different periods of laboratory acclimation, possible intracellular bacterial infection, and food quality. We discuss the interval for feeding rate, which could be taken as a characteristic for control animals – *i.e.* interquartile range – and suggest it as a benchmark for feeding rate of *P. scaber* in a laboratory single-species test.

Methods

Model organisms

Terrestrial isopods (*Porcellio scaber*, Isopoda, Crustacea) were collected between June 2006 and June 2013 at different locations in Slovenia, which were all considered to be unpolluted. Prior to experiments, the animals were kept in a terrarium filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*), at a temperature of 20 ± 2 °C and a 16:8-h light:dark photoperiod. They were acclimated to laboratory conditions for at least 14 days before the start of the experiment. Adult animals of both sexes, weighing more than 25 mg, were used in the experiments.

Experimental design (Drobne and Hopkin 1994, Novak et al. 2012)

Hazelnut leaves were dried at room temperature, cut into pieces weighed and placed in a Petri dish. One animal was placed in each dish together with two to four pieces of leaves, which were the isopods only food source. Petri dishes were kept in a large glass container under controlled conditions in terms of humidity (≥80%), temperature (21 ± 1 °C) and light regime (16:8-h light:dark photoperiod). After 14 days, the leaf remnants were removed, dried and weighed.

Feeding parameter

Feeding rate per day (*FR*) was calculated as the amount of food consumed divided by the isopod fresh weight at the beginning of exposure (*IW*) per day.

Data analysis

The data presented here were obtained from 60 different experiments conducted with 594 animals between 2006 and 2013. These specimens served as control animals in different feeding experiments. All experiments were conducted following the same exposure protocol, and standard operational procedure (Drobne and Hopkin 1994, Novak et al. 2013). The observed variables of the control animals, i.e. the weight of the animal at the beginning of experiment and the feeding rate per day, were analysed and tested using parametric and non-parametric statistical methods. The distribution of each variable was determined using the Kolmogorov-Smirnov test. The number of classes (*k*) for both variables was defined by Sturges rule for skewed data:

$$k = 1 + log_2(n) + log_2\left(\gamma_1 + \sqrt{\frac{n}{s}}\right),$$

where n is the number of observations, γ_1 is the coefficient of skewness, and s is standard deviation of observed data. Correlation between observed variables was tested by a t-test. The interquartile range of feeding rate per day was chosen arbitrarily as a reference interval for the control animals. Interquartile range defines the central 50% of sample. The interquartile range of observed variables of control animals was estimated by confidence intervals for the first (Q_1) and the third quartile (Q_3) using:

$$j(\cdot) \approx \left[nq - z_{\alpha/2}\sqrt{nq(1-q)}\,\right]$$

$$k(\cdot) \approx \left[nq + z_{\alpha/2}\sqrt{nq(1-q)}\,\right] + 1,$$

where q is the quantile rank of Q_1 (q=0.25) and Q_3 (q=0.75) respectively, $j(Q_1)$ and $j(Q_3)$ were ranks of lower limits of the confidence interval of Q_1 respectively Q_3, $k(Q_1)$ and $k(Q_3)$ were ranks of upper limits of confidence interval of Q_1 respectively Q_3. Confidence intervals for quartiles were estimated for risk level at $\alpha < 0.05$, $\alpha < 0.01$ and $\alpha < 0.001$. Analysis and graphical presentations were performed in SPSS 21 and Excel 2013.

Results

Variation of feeding rate per day (*FR*), judging by the coefficients of variation, is twice the variation of the isopod weight (*IW*) at the beginning of the experiment (Table 1). Table 1 and especially Figs 1 and 2 reveal that the observed variables of the populations are not normally distributed (Kolmogornov-Smirnov test: for both variables p<0.001). Figs 1 and 2 show a frequency distribution histogram, polygon of cumulative frequency, as well as the interquartile range (Q_3-Q_1) of animal weight and their feeding rate. Considering the weight of animals, there were less higher-weight animals than the mean weight (Fig. 1). Similarly, there were less animals with higher feeding rate per day then the mean feeding rate per day (Fig. 2). However, the results of the correlation analysis showed that *P. scaber* weight and feeding rate

Table 1. Descriptive statistics for animals (*Porcellio scaber*) from laboratory-feeding experiments.

Statistics	Isopod weight at the beginning of experiment (in mg; *IW*)	Feeding rate per day (in mg/g; *FR*)
Number (*n*)	594	594
Minimum (*min*)	15.800	0.001
Maximum (*max*)	105.200	0.257
Mean (*m*)	44.800	0.055
Variance (s^2)	177.672	0.001
Standard deviation (*s*)	13.329	0.033
Coefficient of variation (*CV*)	0.298	0.599
Skewness (γ_1)	1.033	2.251
Kurtosis (γ_2)	1.879	7.916
First quartile (Q_1)	35.400	0.035
Median (*Me*)	43.050	0.048
Third quartile (Q_3)	52.125	0.068

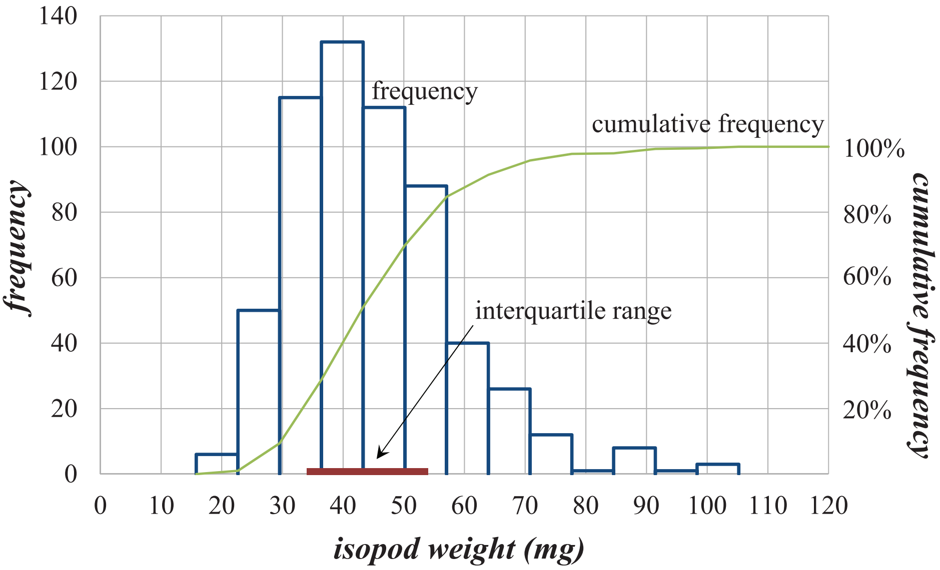

Figure 1. Frequency distributions and interquartile range of *Porcellio scaber* weight at the beginning of the experiment ([34<*IW*<54] mg).

per day were negatively correlated (Fig. 3) indicating that higher-weight animals consumed less food per day. The Pearson coefficient of correlation was significant at $p<0.001$ (T=-10.03).

Table 2 shows confidence intervals for the first (Q_1) and the third (Q_3) quartile for analysed variables and for different probability Pr (Pr=1-α) assuming that Q_1 and Q_3 in the samples are normally distributed.

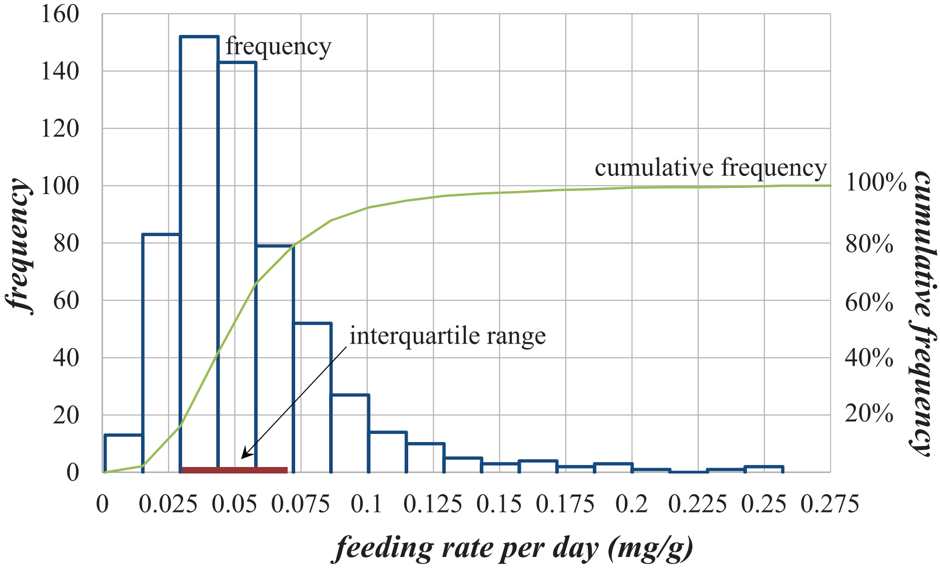

Figure 2. *Porcellio scaber*: Frequency distributions and interquartile range of feeding rate per day ([0.03<*FR*<0.07] mg/g).

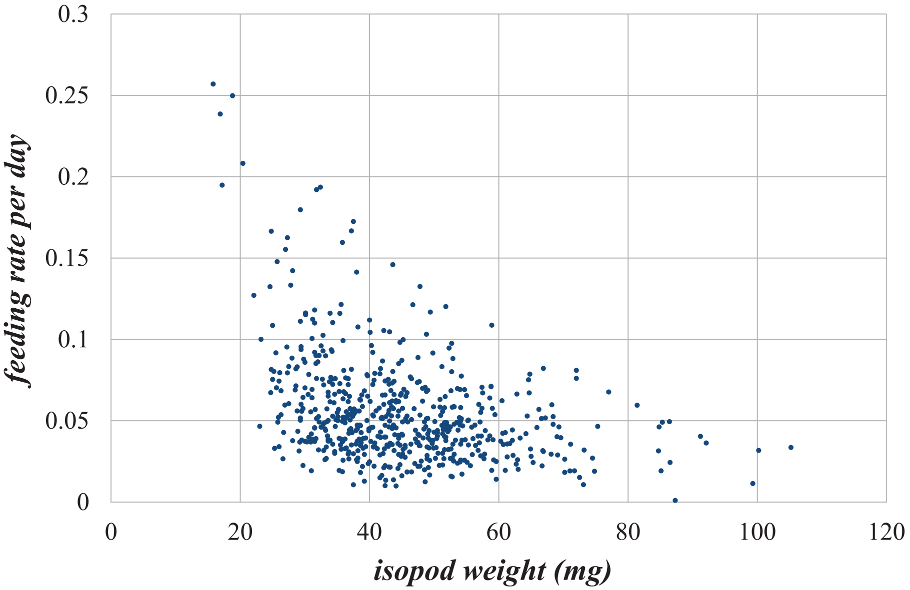

Figure 3. Scatter graph of *Porcellio scaber* weight and feeding rate per day (r=-0.38; p<0.001).

In the case where the probability Pr=0.99 was chosen, feeding rate per day (*FR*) of the central 50% of the control population was situated in the interval [0.032,0.073] mg/g; Pr[0.032<*FR*<0.073]=0.99. In addition, if probability was lower

Table 2. *Porcellio scaber*: Confidence intervals for Q_1 and Q_3.

Isopod weight at the beginning of experiment (in mg; *IW*)	**Feeding rate per day (in mg/g; *FR*)**
$\Pr[34.28 < Q_1 < 36.40]=0.95$	$\Pr[0.033 < Q_1 < 0.037]=0.95$
$\Pr[50.93 < Q_3 < 53.14]=0.95$	$\Pr[0.065 < Q_3 < 0.071]=0.95$
$\Pr[33.78 < Q_1 < 36.80]=0.99$	$\Pr[0.032 < Q_1 < 0.037]=0.99$
$\Pr[50.43 < Q_3 < 54.00]=0.99$	$\Pr[0.062 < Q_3 < 0.073]=0.99$
$\Pr[33.48 < Q_1 < 37.01]=0.999$	$\Pr[0.032 < Q_1 < 0.038]=0.999$
$\Pr[49.83 < Q_3 < 54.20]=0.999$	$\Pr[0.062 < Q_3 < 0.074]=0.999$

(Pr=0.95), the result for the interquartile range of feeding rate per day was similar ($\Pr[0.033<FR<0.071]=0.95$). Therefore, the central 50% of values of feeding rate per day were in the interval $[0.03<FR<0.07]$ mg/g.

The reference values for our laboratory control *P. scaber* was between 0.03 and 0.07 mg/g of feeding rate per day ($[0.03<FR<0.07]$ mg/g) when their weight at the beginning of the test was in the range between 34 and 54 mg ($[34<IW<54]$ mg) (Figs 1–2).

Discussion

Terrestrial isopods of the species *Porcellio scaber* are among the most frequently used species in (eco)toxicity testing, and feeding parameters are among more relevant ecotoxicity endpoints (Drobne 1997). They indicate organism level responses and potential ecological implications. A significant obstacle of using feeding parameters as an endpoint is their high variability already in a control population (Drobne and Hopkin 1994). To overcome this shortage, we propose reference benchmark values for feeding rate, which we define as a characteristic values for control animals. According to our results, the feeding rate of *P. scaber* per day of the central 50% of the control population was between 0.03 and 0.07 mg food/g animal/day. The deviation from these values might indicate an altered physiological state of animals.

Benchmark values for measured parameters in control animals allow direct comparison of measurement results in proficiency testing (round robin testing) performed by different laboratories (ASTM E1301-95). In addition, benchmark values for control animals could also be used in hazard and risk assessments to define data quality (Hobbs et al. 2005).

The data for feeding rate were not-normally distributed and nonparametric statistics must be used for further analysis. Not-normal data distribution can be explained by the fact that some animals could eat less or even stop eating, but tested animals could not eat above a certain limit. The coefficient of variation allowed us to compare the variation of different data. When we correlated feeding and isopod weight, it appeared that the correlation is relatively low suggesting that feeding behaviour of animals depends not only on weight but also on other parameters, which have not been tested yet or are even unknown. Our results suggest that data for feeding rate have higher variability as data for isopod weight.

Information on both technical and biological variance is of major importance in interpretation of the results of toxicity testing and in assessing their quality. Technical variance is attributed to performance of experiments and experimental conditions, while the biological source of variance is an inherited biological variability of the measured parameter. In our tests, most of the variance originated from the biological variability, and the technical variance was presumed to be low due to controlled and constant experimental conditions. Our data confirm that variance is an intrinsic characteristic of feeding parameters. Analysing and understanding the variance is of significant importance in the decision-making process used to set proper safety factors (Warren-Hicks et al. 2000).

In a single species laboratory test with *P. scaber* several endpoints are commonly assessed. These are biochemical biomarkers, histopathological changes, behavioural responses and physiological alterations as well as different organism level responses including moulting, mortality and growth. The selected biomarkers vary in sensitivity, ease of observation, reproducibility, repeatability and ecological importance (Jemec et al. 2010). Conventional measures of toxicity such as growth, reproduction and life-cycle have high ecological relevance, but are difficult to assess under controlled laboratory conditions. Biochemical endpoints are very sensitive and respond quickly but they have limited ecological relevance (Jemec et al. 2010). Feeding parameters are intermediate between these endpoints. They are less sensitive as biochemical biomarkers, but still are readily reproducible and repeatable under controlled laboratory conditions (Drobne and Hopkin 1994). Literature data provide evidence on reduced feeding parameters after exposure of *P. scaber* to metals, biocides or veterinary drugs in a dose-dependent manner (for summary see: Novak et al. (2012, 2013). However, in case of the pesticide diazinon, an increased but dose-independent feeding rate was reported (Stanek et al. 2006). After exposure of *P. scaber* to silver nanoparticles in our laboratory, the feeding rate increased, but again not in a dose-dependent manner. These data indicate that feeding behaviour dynamics in isopods can be changed by different food additives. We suggest that in subchronic exposures, the benchmark values for feeding parameters as well as the coefficients of data variability may support the elaboration of lowest observed adverse effect concentrations, and data interpretation should not be based solely on statistical tests of significance. In addition, this approach could also improve significantly the quality of data.

Feeding rate of terrestrial isopod *P. scaber* either decreases or increases due to food additives. The significance of this change is difficult to assess when a parameter has high background variability. We suggest using reference, benchmark data for feeding parameters and coefficients of variation for feeding rate to: (a) discriminate between animals with altered feeding parameters with respect to a control in addition to existing statistical tests; (b) assess the data quality in future experiments by comparing of the coefficient of variation and the established range for feeding parameters in control animals. In addition, establishing also reference, resting values for other parameters analysed in toxicity tests could increase the relevance of toxicity data such as ECx, LOEC, NOEC, and could assist significantly in quality control of the data.

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Agonistic behaviour in juvenile southern rock lobster, *Jasus edwardsii* (Decapoda, Palinuridae): implications for developing aquaculture

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Abstract

The Southern rock lobster, *Jasus edwardsii*, is a temperate species of spiny lobster with established well managed fisheries in Australia and New Zealand. It has also been under consideration as a species with aquaculture potential. Agonistic behaviour has important consequences under aquaculture conditions that encompass direct effects, such as damage or death of protagonists, and indirect effects on growth that relate to resource access, principally food and refuge. This study aimed to identify and characterize behaviours and to make a preliminary investigation of their occurrence under tank culture. Juvenile *Jasus edwardsii* were examined in a flow-through seawater system using a remote video camera system. Twenty-nine behaviours were divided into three sub-groups: aggressive (11), avoidance (6) and others (12). Aggressive behaviours included attacks, pushing, lifting, clasping and carrying an opponent. Avoidance behaviours included moving away in a backwards-, forwards- or side-stepping motion as well as with more vigorous tail flips. These behaviours were components of twelve behavioural groups that described contact, attack and displacement between individuals. Activity was crepuscular with two clear peaks, one in the morning and the other in the evening. The occurrence of behavioural groups was not different between the morning and evening. The frequency of aggressive behaviours was not affected by changes made to stocking density or access to food. The implications of agonistic behaviours are discussed further in relation to developing aquaculture.

Keywords

Spiny lobster, aquaculture, con-specific, behaviour, inter-individual interaction, growth depensation

Introduction

Spiny lobster species are found around the world from tropical to temperate seas, they are important in relation to conservation, fisheries and aquaculture (Green et al. 2010). The Southern rock lobster, *Jasus edwardsii*, is a temperate species with established and well managed fisheries in Australia and New Zealand (Holland et al. 2005, Linnane et al. 2012, Linnane et al. 2013). It too has also been under consideration as a species with aquaculture potential (McKoy 1979, Crear et al. 2000, Simon and James 2007). Some advantages relate to reported behavioural characteristics that make it amenable to group culture and arguably more suitable for aquaculture than other lobster species, particularly clawed lobsters (McKoy 1979, Huber and Kravitz 1995, Thomas et al. 2003).

Clawed lobsters exhibit an array of aggressive behaviours, they readily fight to establish and maintain dominance hierarchies and therefore exhibit behaviours that are not ideal for high productivity under typical intensive communal aquaculture systems (Karavanich and Atema 1998, Kravitz 2000, Gherardi et al. 2010). Agonistic behaviour has important consequences under confined aquaculture conditions that relate to access to resources, principally food and refuge, and that encompass direct effects, such as damage or death of protagonists, and indirect effects on growth and health (Thorpe and Huntingford 1992, Drengstig and Bergheim 2013). Hierarchical social structures often lead to feeding hierarchies where there is unequal distribution of food between individuals, differences in food intake result in growth depensation (McCarthy et al. 1992, Winberg et al. 1993). Growth depensation is an increase in variance of a size distribution with time due to individuals growing at different rates (Magnuson 1962, Carter et al. 1996), and its management is critical for aquaculture production (Thorpe and Huntingford 1992, Drengstig and Bergheim 2013).

Compared to clawed lobsters (Huber and Kravitz 1995, Drengstig and Bergheim 2013) less is known about the agonistic behaviour of spiny lobsters, especially in relation to aquaculture (Thomas et al. 2003, Moyle et al. 2009). Spiny lobsters exhibit an extensive range of behaviours including agnostic behaviours which are particularly prevalent around obtaining and retaining shelter (Fielder 1965, Berrill 1976, Cobb 1981, Segura-García et al. 2004). Spiny lobsters aggregate in and around shelters in the wild, yet they also compete for shelters and show aggression around them (Childress 2007). The more solitary species of spiny lobsters are considered more aggressive (Childress 2007) and *Jasus edwardsii* , the subject of the current study, appears more gregarious and less aggressive than some other spiny lobsters (Fielder 1965, Berrill 1976, James et al. 2001, Moyle et al. 2009). Chemicals in the urine and physical contact are the important mechanisms mediating social interactions including aggregation, mating, and agonistic behaviours in spiny lobsters (Briones-Fourzan et al. 2008, Horner et al. 2008, Shabani et al. 2009). Agonistic interactions often involve overt aggressive and submissive behaviours, published information on the nature of these interactions in captivity is spread across spiny lobster taxa *Jasus* (Fielder 1965, Thomas et al. 2003) and *Panulirus* (Berrill 1976, Cobb 1981, Segura-García et al. 2004, Moyle et al. 2009). Consequently, the current study aimed to identify and characterize behaviours of *Jasus edwardsii* and to make a preliminary investigation of their occurrence under tank culture conditions and in relation to some key factors that can be manipulated in order to improve lobster growth and productivity.

Methods

Juvenile *Jasus edwardsii* stock were caught as puerulus from the East coast of Tasmania and maintained in 4 m^3 rathbun tanks. Stock and experimental animals were held at 18 °C, and fed on a mixed diet of whole blue mussel, frozen squid and a commercial prawn feed. Lobsters held under standard conditions were used in experiments (Crear et al. 2000). Only the prawn feed was used during the experiments and fed at 1% body weight per day (Thomas et al. 2003). All animals survived and were returned to stock, they were only used once.

Behaviours were examined in two rectangular 25L (width 300 × length 500 × depth 250 mm) tanks that were part of a flow-through seawater system. The flow rate was 7.4 L min^{-1} so that water was replaced approximately 18 times per hour. The system was enclosed by black plastic sheeting to control photoperiod and minimise visual disturbance from external sources. Light was provided from fluorescent tubes and a 12:12 photoperiod used with lights-on at 06:00 and lights-off at 18:00. An infrared light provided illumination for the camera during the dark phase. Each tank system was equipped with a video camera and the tanks were stocked with 5 animals (equivalent to 33.5 individuals per square metre, ind. m^{-2}). The carapace was marked to identify each individual. The floor of each tank was marked with a 100 × 100 mm grid so distance and direction of movements could be assessed more easily.

An initial assessment of activity was made on two groups of five animals that were monitored continuously for seven days. Focal sampling (Altmann 1974, Shelverton and Carter 1998) was used to record activity for the first ten minutes of every hour. The unit of activity was defined as movement equivalent to half the length of the lobster. Peaks of activity occurred in the morning between 07:00 and 10:00 and in the evening between 18:00 and 21:00. Twenty-nine component behaviours were identified and used to define twelve behavioural groups (see Results) including when no behaviour was observed (none).

Four new groups of five animals were used to investigate the occurrence of the behavioural groups. Occurrence was the number of times a behavioural group was observed as the first behaviour exhibited during each period of observation: each occurrence of behavioural unit was given a score of 1, observations were made over five days and data adjusted to one hour of observation (Moyle et al. 2009). The effect of time of day was then analysed using these four groups to compare occurrence of behavioural groups between the morning (AM) and evening (PM) periods. The effects of stocking density and feed availability on the occurrence of behavioural groups were measured in separate experiments. The experiments on stocking density and feeding availability were of a preliminary nature and each used 2 groups (10 animals).

Stocking density compared standard with a higher density: density was doubled from the standard density of 33.5 to 67.0 ind. m^{-2} by dividing the tanks in half (one half remained empty during the experiment). The effect of feed availability was investigated by comparing one with four feeding stations: the standard single feeding station in the centre of the tank or using four feeding stations simultaneously, one in each corner of the tank (the total ration was the same).

For each experiment the same sampling regime was used: eight 15 min time blocks, four from the morning (05:00–06:45) and four from the evening (18:00–19:45) periods of peak activity, were analysed and behavioural group recorded against individuals (occ. h^{-1}). Mean group occurrence was calculated for each behavioural group as total occurrence divided by the number of individuals (five) and standardised to an hour of observation, it was expressed as occurrence per individual per hour (occ. Ind^{-1} h^{-1}). Non-parametric statistical analysis using the Kruskal-Wallis test followed by multiple comparison using all pairwise comparisons was conducted due to preliminary nature of the data and the lack of homogeneous variance according to Levene's test. Statistical analysis was conducted using SPSS Statistics version 22.

Results

Component behaviours

Twenty-nine component behaviours were identified and described; 11 aggressive and 6 avoidance (Table 1) as well as 12 others (Table 2). A selection of line drawings to represent aggressive (Figs 1–4), avoidance (Figs 5–8) and other (Figs 9–12) behaviours are presented.

Behavioural groups

Twelve behavioural groups, including one for no behaviour, were defined. Behavioural groups were divided into four broad categories (Table 3). Low Intensity Contact (LIC) involved brief contact via mutual antennal touching before separation. Medium Intensity Contact (MIC) was the same as LIC with additional contact where the legs of one animal came into contact with the dorsal surface of the other. Overtly aggressive behaviours were divided between Attack and Displacement. During an aggressive interaction the aggressor and the subordinate exhibited different component behaviours (Table 3).

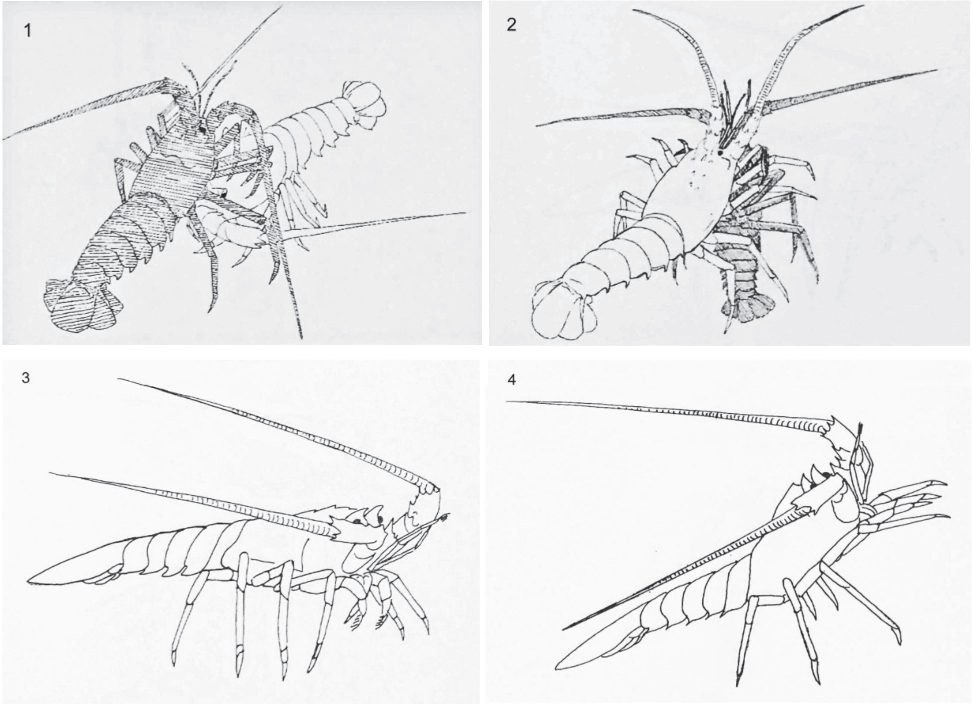
There were statistically significant difference in the occurrence of behavioural groups (Fig. 13). The highest individual occurrence of behavioural groups was for no behaviour, followed by medium intensity MIC1 and MIC3 and by low intensity LIA and LIC1. There were no statistical differences amongst these behavioural groups (Fig. 13a). The same pattern was also apparent when mean group data were analysed (Fig.

Table 1. Component behaviours of *Jasus edwardsii* grouped as aggressive or avoidence behaviour.

Behaviour	**Description**
Aggressive	
Attack (A1)	Moderate contact with opponent (see Fig. 1).
Attack (A2)	Vigorous contact with opponent accompanied by clasp (CL).
Burrow (BU)	Vigorous contact lifting opponent clear of substrate.
Carry (CA)	Transport of opponent.
Chase (CH)	Vigorous pursuit of opponent.
Clasp (CL)	Clasp opponent with first three walking legs (see Fig. 2).
Dislodge (D)	Moderate contact in which aggressor dislodges opponent from position.
Push (P)	Vigorous contact in which aggressor displaces and pushes opponent away from position.
Rise (R1)	Orientate toward opponent and raise anterior by extending walking legs (see Fig. 3).
Rise (R2)	Orientate toward opponent and raise anterior above posterior by extending third, forth and fifth walking legs.
Rise (R3)	Orientate toward opponent and raise anterior high above posterior by extending third, forth and fifth walking legs. Accompanied by waving first two pairs of walking legs at opponent (see Fig. 4).
Avoidance	
Back away (BA)	Moderate backwards movement away from opponent with tail undulation (Fig. 5).
Flee (FL)	Vigorous movement away from opponent propelled by tail flapping (see Fig. 8).
Move away (MA)	Moderate movement away from opponent with no further interaction.
Short distance escape (SDE)	Vigorous movement that separates opponents.
Sidestep away (SSA)	Moderate sideways movement away from opponent (see Fig. 7).
Walk away (WA)	Moderate movement away from opponent (see Fig. 6).

Table 2. Component behaviours of *Jasus edwardsii* other than aggressive or avoidence behaviour.

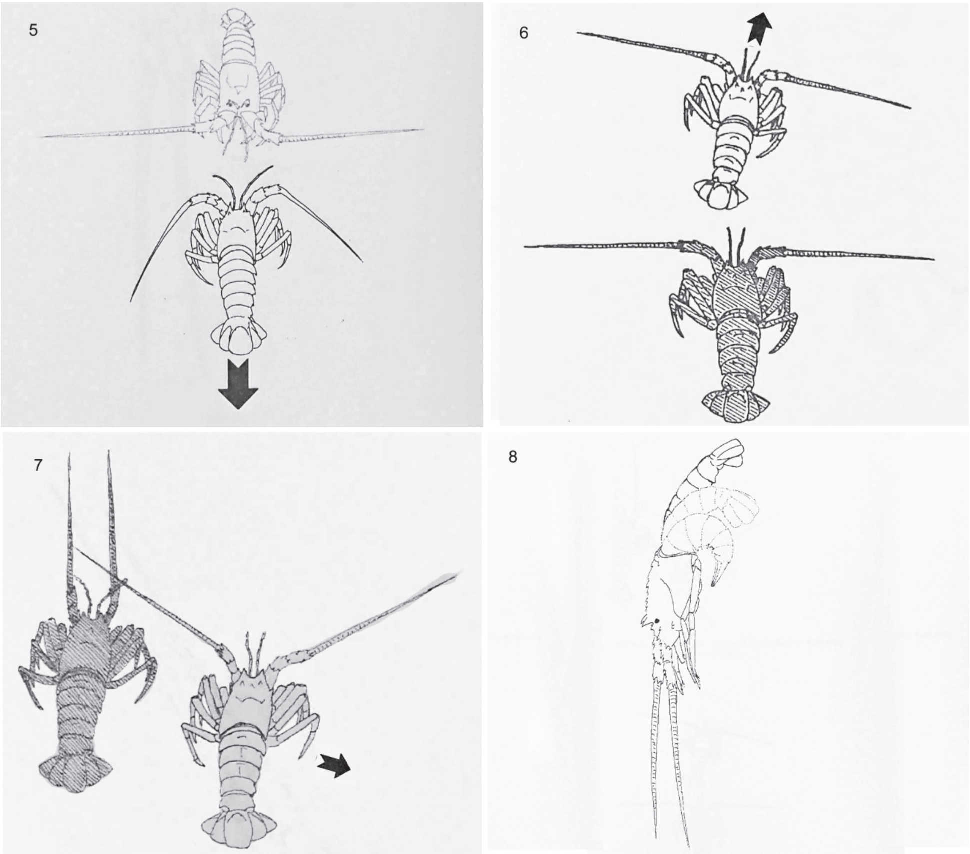
Behaviour	**Description**
Antennal pointing (AN)	Orientation of antennae towards approaching opponent whilst first pair of walking legs raised above substrate.
Antennal touch (AT)	Contact with antennae (see Fig. 11).
Antennule touch (AT2)	Contact with antennules (see Fig. 12).
Approach backwards (APB)	Moderate tail-first movement towards opponent.
Approach forwards (APF)	Moderate head-first movement towards opponent with antennae at 90° to body.
Body touch (BT)	Contact opponent with body, usually aimed at dorsal carapace or head.
Cautious approach (CA)	Slow head-first movement towards opponent (see Fig. 9).
Depress (DE)	Body is flattened on substrate and legs drawn tightly into the carapace.
Face-to-face (FTF)	Head to head orientation, anntennae touching and usually involves contact with antennules.
Quiescence (Q)	Stationary with movement of second and third walking legs to ventillate gills.
Side touch (ST)	Moderate sideways movement and contact with opponent.
Toward (U)	Moderate movement towards opponent (not APB or APF).



Figures 1–4. Selected aggressive behaviours of *Jasus edwardsii*: **1** attack 1 (low intensity) **2** clasp **3** rise 1 (low intensity) **4** rise 3 (high intensity).

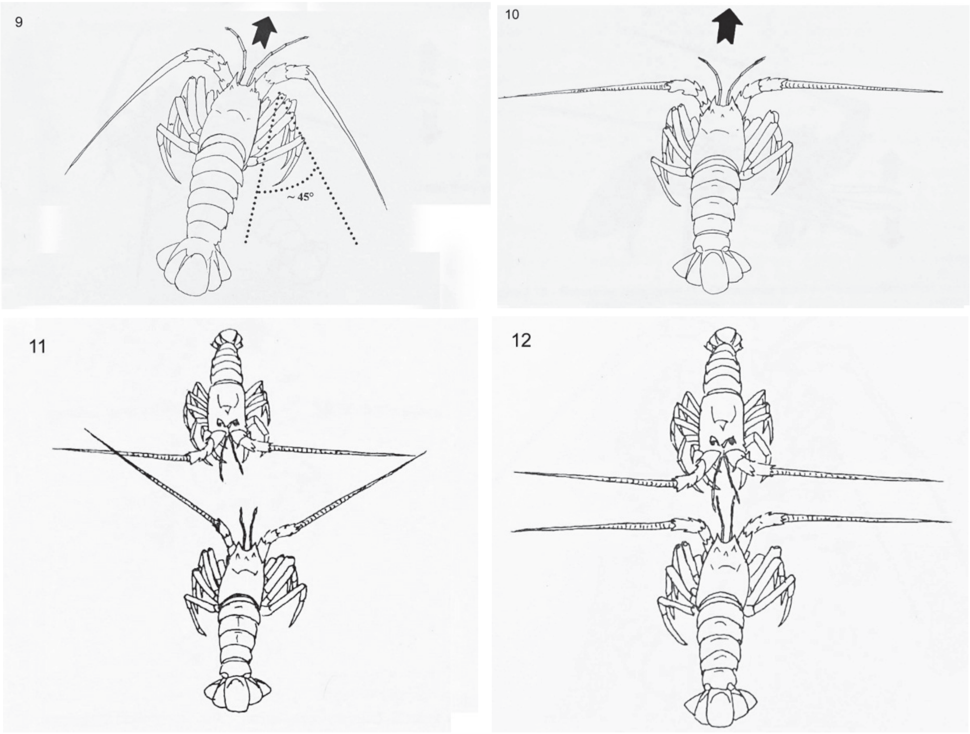
Table 3. Behavioural groups of *Jasus edwardsii* under main categories: low intensity contact, medium intensity contact, attack and displacement. Subordinate behaviour during attack is in parentheses.

Behavioural Group	Behaviours
Low intensity contact (LIC)	
LIC1	Approach forward, antennal touch, walk away.
LIC2	Cautious approach, antennal touch, walk away.
Medium intensity contact (MIC)	
MIC1	Approach forward, antennal touch, body touch, walk away.
MIC2	Cautious approach, antennal touch, body touch, walk away.
MIC3	Approach forward, antennal touch, side touch, walk away.
MIC4	Cautious approach, antennal touch, side touch, walk away.
Attack	
LIA, Low intensity attack	Cautious approach, rise 2, antennal touch, attack 1, (flee), walk away.
MIA, Medium intensity attack	Cautious approach, antennal touch, (back away), chase, rise 2, antennal touch, attack 2, clasp, (flee).
HIA, High intensity attack	Approach forward, rise 3, attack 2, clasp, (short distance escape), chase, rise 3, clasp, (short distance escape), (flee).
Displacement	
FD, Forced displacement	Burrow, carry, dislodge, walk away.
D, Dislodge	Approach forward, side touch, push, walk away.



Figures 5–8. Selected avoidance behaviours of *Jasus edwardsii*: **5** back away **6** walk away **7** side step away **8** flee.

13b). These data considered the number of types of behavioural groups averaged across all five individuals in a tank. Other than exhibiting no behaviour, the predominant component behaviours were approach forward (Fig. 10), antennal touch (Fig. 11), side or body touch and walk away (Fig. 6). The occurrence of LIA also included rise 2, attack 1 (Fig. 1) by the aggressor and flee (Fig. 8) by the recipient (Table 3). Conversely, behaviours unique to medium and high intensity attack, such as chasing and clasping, as well as forced displacement and dislodge were rarely observed. There were no significant differences between morning and evening (Kruskal-Wallis test, n = 4, d.f. = 1, P=0.744), standard and higher stocking density (Kruskal-Wallis test, n = 4, d.f. = 1, P=0.616) or using four rather than one feeding station (Kruskal-Wallis test, n = 4, d.f. = 1, P=0.724). Thus, there was no evidence that changes made here to the standard density and feeding management had any influence on behaviours. The pattern of behavioural groups was generally similar to that presented above with LIA, LIC1, MIC1 and MIC3 having the highest occurrence. However, replication was insufficient for further analysis.



Figures 9–12. Selected other behaviours of *Jasus edwardsii:* **9** cautious approach, **10** approach forward **11** anntennae touch **12** antennule touch.

Discussion

Agonistic behaviour has been divided into approach- and avoidance-orientated behaviour (Huber and Kravitz 1995). Clawed lobsters exhibit extensive agonistic behaviours, they are aggressive, solitary and cannibalistic when cultured together (Drengstig and Bergheim 2013). The American lobster, *Homarus americanus*, has an extensive repertoire of aggressive behaviours that include use of the large claws to signal, touch, manipulate and damage opponents. An important aspect of aggressive bouts is to displace opponents, this includes attempting to lift and turn them on their backs as well as pushing or pulling them away from the substrate (Huber and Kravitz 1995). In contrast, many spiny lobsters are reported to be gregarious and only vulnerable to cannibalism following moulting and might, therefore, be predicted to exhibit behaviours that facilitate co-habitation (Childress 2007). However, in wild *Jasus lalandei* this was a function of the capacity of the shelter and smaller individuals were actively displaced by larger individuals (Fielder 1965). Furthermore, in aquaculture the local tank environment is a critical factor that influences behaviour with consequences on growth and production (Thomas et al. 2003, Moyle et al. 2009). The current research aimed to make a detailed study of *Jasus edwardsii* behaviours and behavioural groups in order to provide a basis for understanding the effects of the aquaculture environment.

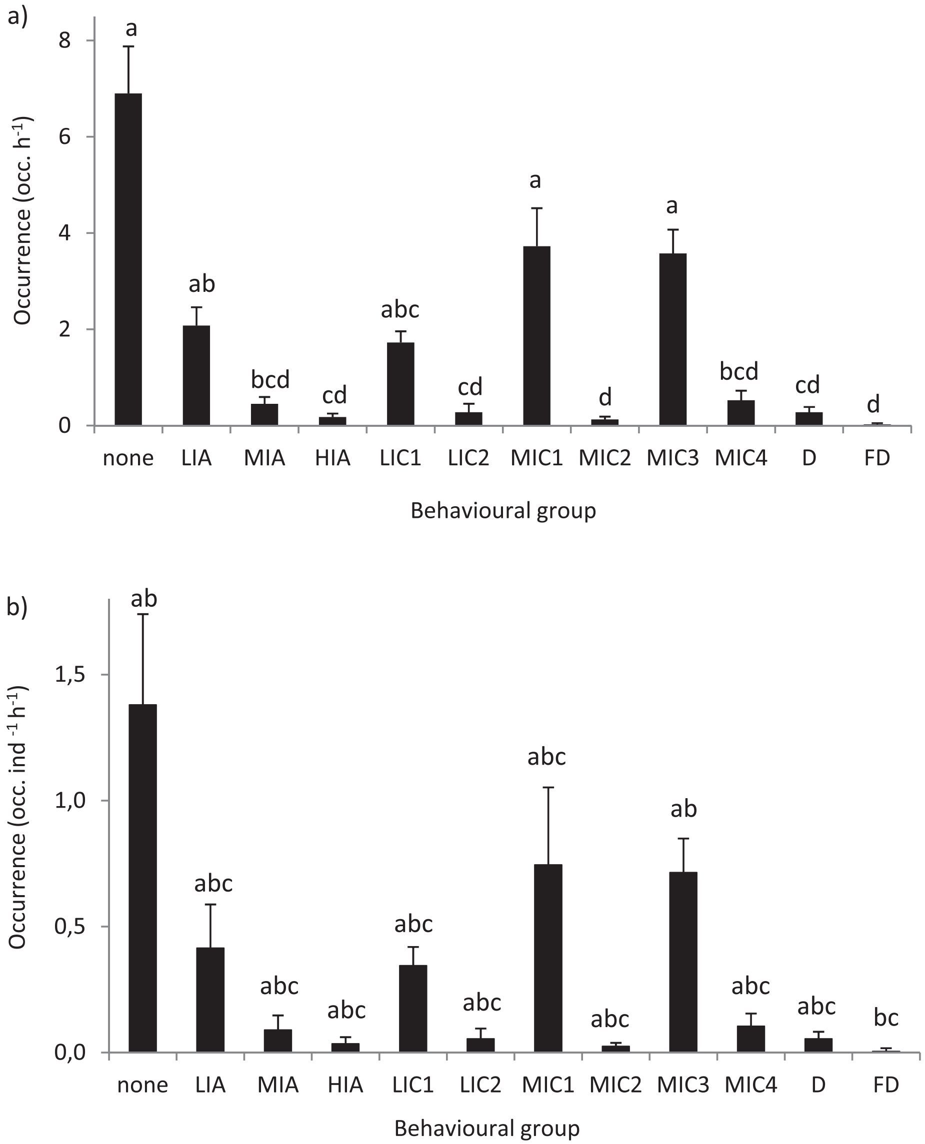


Figure 13. Occurrence of *Jasus edwardsii* behavioural groups as a) mean (± SE) individual occurrence (occ. h^{-1}) and b) mean (± SE) group occurrence per individual per hour (occ. ind^{-1} h^{-1}). Individual occurrence (a): significant difference amongst behavioural groups (N = 20, P<0.001, Kruskal-Wallis Test). Group occurrence (b): significant difference amongst behavioural groups (n =4, P<0.001, Kruskal-Wallis Test). No significant different amongst means with same letter.

Behaviours

Thirty-two behaviours were observed in the spiny lobster *Panulirus cygnus* under a variety of situations in the wild or during captivity and sixteen related to aggressive interactions including five linked to subordinate responses (Cobb 1981). In bouts between pairs of clawed lobster, *H. americanus*, seventeen behavioural components were recognised (Huber and Kravitz 1995). “Approach” and “lunge” described slow and rapid approach behaviour, respectively, and corresponded to cautious and forward approach used in the current study. Moderate avoidance behaviour “retreat” was represented in more detail in the current study by move away, back away, walk away and sidestep away whereas the more vigorous “tailflip” matched flee. Avoidance behaviours exhibited by *J. edwardsii* were also characteristic of *P. cygnus* (Cobb, 1981). In contrast to *H. americanus*, *J. edwardsii* exhibited several forms of contact, termed touch, between individuals that were part of low and medium intensity contact behavioural groups and that were not obviously agonistic. When alone *P. cygnus* would sometimes straighten and hold their legs horizontally and suddenly settle in a behaviour termed splay (Cobb 1981), this was somewhat similar to “depress” in *J. edwardsii* except the legs were drawn in. All these species orientated towards an aggressor or on-coming animal and raised their anterior body up. The large claws of *H. americanus* are an obvious difference between the two types of lobster and were used extensively in combat and seven behaviours identified. *J. edwardsii* exhibited different aggressive behaviours that involved displacement through pushing, burrowing and carrying as well as clasping the opponent. The long antennae are used aggressively and defensively by spiny lobsters (Fielder 1965, Cobb 1981), in the present study all eight low and medium intensity behavioural groups included antennal touch as a component behaviour. Interestingly, the high intensity attack did not involve antennal touch and escalated rapidly to attack. This suggested that the presence of chemical signals from urine may have played an important part in communicating social status. In *P. argus* urine is important in reducing the levels of aggression in formed hierarchies; dominant animals increase urine release when engaged in interactions while subordinate animals do not (Shabani et al. 2009). The development of effective hatchery rearing of lobsters, whether on-grown or raised entirely in the hatchery, presents an opportunity for re-stocking. One relevant issue is the behaviour of hatchery raised lobsters to predators (Oliver et al. 2006, Mislan and Babcock 2008), the current research did not address this but it is clearly of importance if animals are to be released into the wild. Research suggests that wild caught post-puerulus that were on-grown juveniles retain a level of plasticity in behaviour and around assessing risks of predation and shifting to nocturnal activity to decrease predation risk (Oliver et al. 2006).

Stocking density and feed availability

Density has some significant effects on behaviour of spiny lobsters under culture conditions, frequency of occurrence was in the order of 10 time lower for small post-puerulii *P. cygnus* stocked at the lowest density of 30 individuals per m^2 compared with at 60 to 150 individuals per m^2 (Moyle et al. 2009). In the current study doubling stocking density did not appear to have any effect on the occurrence of any behavioural groups, including the groups that contained attack behaviours. The standard density used here was similar to 35 individuals per m^2 and doubling it to 67 was within a range that appears appropriate for tank populations of *J. edwardsii* (James et al. 2001). For example, our standard density was similar to that used in a long-term eight month growth experiment on *J. edwardsii* (Simon and James 2007) in which survival was over 80% for animals fed good quality diets. This is likely to relate to the gregarious nature of *J. edwardsii* which would allow this species to adjust to a higher density without any increase in overt aggression. Although not statistically different, there was a numerical increase in the occurrence of no behaviour (none), this meant that at the higher density the number of animals that did not exhibit any behaviour doubled. Although this possible density-effect requires further investigation, *J. edwardsii* may have behavioural mechanisms that will allow them to be held communally at high densities. Excellent survival (>90%) was achieved at up to 200 ind. m^2 in small *J. edwardsii* juveniles (2 g), although weight gain over 118 days increased by as much as 80% when stocking density was decreased from 200 to 50 individuals per m^2 (James et al. 2001). In the present study, the occurrence of aggressive behaviours was not affected by increasing access to feed. Further study will be required to determine whether *J. edwardsii* would attempt to defend a food source but their foraging behaviour in the wild and observations made on feeding behaviour in tanks would suggest this is not normally the case. Food type may also influence the response, with whole or half-shell blue mussels being more defendable than small pellets, as well as having a higher nutritional value than commercial prawn feeds currently fed to *J. edwardsii* juveniles (Crear et al. 2000, Simon and James 2007).

Aquaculture

Pilot aquaculture studies support the conclusion from the current study that agonistic behaviours have a low occurrence in *J. edwardsii* (Bryars and Geddes 2005, Simon and James 2007). A significant consequence of agnostic interactions is the maintenance of social hierarchies that can translate into feeding hierarchies, unequal distribution of food and differential growth rates amongst individuals (McCarthy et al. 1993). Growth depensation is the increase in variation in individual size within a group of animals and it provides an informative measure that describes changes in production (Koebele 1985). Simon and James (2007) housed small juvenile lobsters (2.85 g) in specially adapted cages in the sea and fed them either green mussels, moist feed, pelleted dry feed or with no extra feed and relied on any available wild prey and bio-fouling. Over eight months fed lobsters increased in weight by up to 10 times with high survival. There were differences in final weight amongst the feeding regimes; in descending order of, green mussels (×10), pelleted feed (×6), moist feed (×4) and no additional feed (×2). Further analysis showed little change in the weight distribution when fed mussel and pelleted feeds but a doubling of the coefficient of variation with no extra feed. Thus, access to adequate feed appeared to determine the amount of growth depensation and suggested that feeding hierarchies were weak when the amount and nutritive value of a feed were both high. Survival was high and there were no instances of limb damage (C. Simon, personal observation), conspecific attacks being one possible cause of limb damage. In a 12 month holding experiment only 2 out of 52 large 700 g lobsters showed any increase in limb damage that could have been directly attributable to agonistic interactions although tail fan damage was significant (Lorkin et al. 1999). Broadly similar observations were made in later experiment using better feeds and in which more animals moulted (Bryars and Geddes 2005). The long term holding experiments showed that *J. edwardsii* can co-exist in the same enclosed space without any extreme consequences, direct or indirect, on growth and survival and provide evidence of the species having the behaviours that facilitate this. Further, even with small groups of five individuals as used in the current study, the high occurrence of no behavioural groups (none) and the absence of contact or attack behavioural groups helps explain why the long-term holding experiments were relatively successful. The main issue is clearly related to feed formulation and nutrition and not communal holding of rock lobsters (Crear et al. 2000, Thomas et al. 2003).

Conclusion

Twenty-nine behaviours were detected and characterised into three sub-groups in this study: aggressive (11), avoidance (6) and others (12). Among the behaviours recorded, the occurrence of medium and high intensity attacks and displacement events were minimal. This suggested *J. edwardsii* juveniles are highly gregarious in culture, even at relatively high stocking densities (67 individuals per m^2). Further research into characterising the agonistic behaviours of various spiny lobster species with potential for commercial aquaculture is warranted. Better characterisation of agonistic behaviours in culture will assist in testing and improving husbandry management strategies which may include the use of chemical cues, better feed management and shelter designs.

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Multiple parasitic crustacean infestation on belonid fish *Strongylura strongylura*

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Abstract

Simultaneous multiple infestation of parasitic crustacean species involving a cymothoid isopod, *Cymothoa frontalis* Milne Edward, 1840 and four species of copepods such as *Lernanthropus tylosuri* Richiardi, 1880, *Caligodes lacinatus* Kroyer, 1863, *Bomolochus bellones* Burmeister, 1833 and *Dermoergasilus coleus* Cressey & Collette, 1970 was frequently noticed on spot-tail needlefish, *Strongylura strongylura* (Belonidae) captured from the Malabar coast (Kerala, India) during the period from April 2011 to March 2012. All the 43 fishes (*Strongylura strongylura*) collected, were under the hyper-infection with parasitic crustaceans; a total of 388 parasitic crustaceans including 57 *C. frontalis*, 252 *L. tylosuri*, 31 *C. lacinatus*, 24 *B. bellones* and 32 *D. coleus* were recovered from the host fish. 4 members (9.30%) of host fish were under quadruple parasitism, in two different combinations. Seventeen (39.53%) host fishes showed triple parasitism and 20 (46.51%) members exhibited double parasitism, with four and five parasitic combinations respectively. Remaining two (4.65%) fishes were parasitized only by the copepod, *L. tylosuri*. The infestations by all recovered parasitic crustaceans were highly site specific. The damage caused by the parasitic crustaceans was also discussed.

Keywords

Isopod, Copepod, quadruple parasitism, triple parasitism, double parasitism, *Strongylura strongylura*

Introduction

Parasitic diseases in fish seriously limit aquaculture production and its economic viability; knowledge of fish parasites, therefore, is an essential requirement for successful aquaculture (Elshahawy and Desouky 2012). Parasitic crustaceans infesting the fishes generates considerable parasitological interest and is of great economic importance inasmuch as it could adversely affect the health of food fishes (Karlsbakk et al. 2001, Johnson et al. 2004, Trilles et al. 2011, 2012, Aneesh et al. 2013a). They feed on the host mucus, tissues and blood and inflict fatal injuries through secondary infection (Margolis et al. 1975, Margolis and Kabata 1988, Oktener and Sezgin 2000). With the increased development of semi-intensive and intensive, freshwater, brackish water and marine aquaculture, the importance of the study of parasitic crustaceans as a major pest has become more evident inviting the attention of many investigators throughout the world (Johnson et al. 1996, 2004, Williams and Williams 1998, Izawa and Choi 2000, Ho et al. 2000, Karlsbakk et al. 2001, Hadfield et al. 2010, 2011, 2013).

Most of the parasitic crustaceans belong to Isopoda, Branchiura and Copepoda (Margolis et al. 1975, Oktener and Sezgin 2000). Cymothoids are oligoxenous isopods and often induce deleterious effects on the host (Overstreet 1978, Kabata 1985, Trilles and Hipeau-Jacquotte 1996, 2012, Aneesh et al. 2013a). Significant proportion of the parasitic copepods is known to be parasitizing fishes (Kabata 1979, Love and Moser 1983, Hogans and Dadswell 1985, Pillai 1985, Benz 1986, Oldewage and Smale 1993, Benz et al. 2003, Cheng et al. 2009, 2011, Ho et al. 2010, El-Rashidy and Boxshall 2010, 2012). Indian fishes have been shown to possess high rate of susceptibility for parasitization by isopods and copepods as well (Pillai 1985, Aneesh et al. 2012, 2013b, Trilles et al. 2011, 2012, Helna et al. 2013, Vijayakumar et al. 2013, Bharadhirajan et al. 2013).

Reports are scanty on the simultaneous occurrence of multiple parasitism involving exclusively parasitic crustaceans. Daniel and Rao (1967) and Daniel and Premkumar (1967) reported the simultaneous infestation of flying fish (*Cypselurus speculiger*) by a copepod, *Pennella* sp. and the cirriped, *Conchoderma virgatum*. Hewitt (1979) and Benz et al. (2003) observed the multiple infestation of Pacific white shark (*Carcharodon carcharias*) by 5–8 different siphonostomatoid (copepod) species. In India, incidence of double parasitism involving the isopod, *Nerocila phaiopleura* and the copepod, *Lernaeenicus sprattae* was reported in anchovy fish, *Stolephorus commersonii* (Rajkumar et al. 2006). Another Indian fish (*Hemiramphus far*) also showed simultaneous infestation by the isopod, *Mothocya plagulophora* and the copepod, *Lernaeenicus hemiramphi* (Gopalakrishnan et al. 2010). The simultaneous multiple infestations by four parasitic crustacean species on banded needle fish, *Strongylura leiura* was recently reported by Aneesh et al. (2013b).

The present study reports the frequent occurrence of double and triple parasitism and also the few incidence of quadruple parasitism exclusively by the species of parasitic crustaceans including isopod and copepods on the host fish, *Strongylura strongylura* (Belonidae) distributed along the Malabar coast (Kerala, India).

Materials and methods

The present study was conducted during the period from April 2011 to March 2012. Living or fresh fishes, *Strongylura strongylura* were collected from the Ayyikkara fish landing center (Lat. 11°51'N, Long. 75°22'E, Malabar coast, Kerala, India). Soon after collection, the fishes were taken to the laboratory and were examined various parts of the body (such as the general body surface, the lateral line region, base of the pectoral fin, posterio-ventral side, branchial cavity, beak, gill filament, inner wall of the operculum etc.) thoroughly for the presence of parasitic crustaceans using hand lens. Recovered parasitic crustaceans were removed from the host and preserved in 70% ethanol for further detailed examination. The identification was performed, using a dissection microscope and a stereo microscope Leica-S6D, according to Milne Edwards (1840), Cressey and Collette (1970) and Pillai (1985). The prevalence (P) and mean intensity (I) was calculated according to Margolis et al. (1982) and Bush et al. (1997). The host nomenclature and fish taxonomy were done according to Fish Base (Froese and Pauly 2013).

Voucher specimens of all parasites, collected by Aneesh, Helna and Sudha, from the fish, *Strongylura strongylura*, were deposited in the Parasitic Crustacean Museum, Crustacean Biology Research Laboratory, Sree Narayana College, Kannur, Kerala, India. Abbreviations used: PCM – Parasitic Crustacean Museum, Crustacean Biology Research Laboratory, Sree Narayana College, Kannur, Kerala, India; LT – Total length.

Cymothoa frontalis (Milne Edward, 1840): Juvenile (LT. 8 mm) (PCM N° CF-07),13 April 2011; Transitional (LT. 20mm) (PCM N° CF-08), 25 April 2011; Ovigerous female (LT. 26 mm) with manca larva in the brood pouch (PCM N° CF-09), 12 July 2011; 30 Manca larva released by the specimen PCM N° CF-09 (3.4 mm), (PCM N° CF-10), 12 July 2011; Male (LT. 13 mm) (PCM N° CF-14), 08 January 2012.

Caligodes lacinatus (Kroyer, 1863): Ovigerous female (LT, 10.3 mm) with egg sac (PCM N° *Cl*-09), 07 May 2011; ovigerous female (LT, 6 mm) without egg sac (PCM N° *Cl* -13), 23 July 2011.

Lernanthropus tylosuri Richiardi, 1880: Ovigerous female (LT, 5.5 mm) with egg sac (PCM N° *Lt*-01), 07 May 2011; ovigerous female (LT, 5.5 mm) without egg sac and a male (LT, 1.8 mm) clinging on ovigerous female (PCM N° *Lt*-13), 23 March 2012.

Bomolochus bellones Burmeister, 1833: Ovigerous female (LT, 1.9 mm) with egg sac (PCM N° *Bb*-09), 18 June 2011; ovigerous female (LT, 1.8 mm) with egg sac (PCM N° *Bb*-11), 23 June 2011; ovigerous female (LT, 1.4 mm) without egg sac (PCM N° *Bb*-12), 23 June 2011.

Dermoergasilus coleus (Cressey & Collette, 1970): Ovigerous female (LT, 0.6 mm) with egg sac (PCM N° *Dc*-05), 19 July 2011; ovigerous female (LT, 0.5 mm) without egg sac (PCM N° *Dc*-12), 19 January 2012.

Results

Forty three *Strongylura strongylura* (Fig. 1A) collected during April 2011 to March 2012, were found to be infested with five species of parasitic crustaceans. Recovered parasitic crustaceans were identified as cymothoid isopod, *Cymothoa frontalis* (Milne Edward, 1840) and four copepods (such as *Lernanthropus tylosuri* Richiardi, 1880, *Caligodes lacinatus* Kroyer, 1863, *Bomolochus bellones* Burmeister, 1833 and *Dermoergasilus coleus* (Cressey &Collette, 1970) (Tables 1, 2 and 3) (Figs 1B–I).

Among the five parasitic crustaceans recovered from *Strongylura strongylura*, the *Lernanthropus tylosuri* exhibited highest prevalence (81.4%); out of 43 fish (*S. strongylura*) observed 35 member were found to be infested with this lernanthropid copepod (Figs1F and 2A). A total of 252 (218 females and 34 males) members of *L. tylosuri* were recovered from 35 infested fishes and the intensity being 7.2 (Table 2). All females were reproductively active, evidenced by the presence of growing ovaries and/or egg sacs. The recovered males were not independent, but found to be in a clinging/copulatory position, attaching the genital segment of the females with their maxilliped. *L. tylosuri* shows strict site specificity by infesting only the gill filament of the host.

Among the recovered parasitic crustacean species, the isopod *Cymothoa frontalis* exhibited the second highest prevalence (74.42%) (Table 2; Figs 1B–E and 2A) by infesting 32 (out of 43) host fishes (*S. strongylura*); the intensity being equal to 1.78 (Table 2). Of 57 members of *C. frontalis*, there were 21 males and 24 females, 8 juveniles and the remaining 4 were under the transitional phase. Fifty members of this cymothoid parasite were appeared in pairs with three different combinations during their infestation on the fish (*S. strongylura*) such as male–female (18+18=36), juvenile–juvenile (4+4=8) and male–transitional stage (3+3=6); invariably, member in the pair being settled at the floor of either of the branchial cavity of the host fish. The remaining unpaired 7 (57–50) members of *C. frontalis*, including 6 females and one transitional stage were also found to be settled the specific site (floor of the host buccal cavity).

The copepod species, *Caligodes lacinatus* was collected from 17 out of 43 *S. strongylura* examined; the prevalence being 39.53%. A total of 31 parasites were recovered from the infested host fishes, the intensity being equal to 1.82 (Table 2; Figs 1H and 2A). All the recovered members of this parasite were exclusively females carrying growing ovaries and egg sacs as well. *C. lacinatus* was found to penetrate the fleshy part of the lower beak of the host fish; swelling and tissue damages were frequently observed at the penetration site.

Fifteen of 43 *S. strongylura* examined were also infested by 24 members of the copepod *Bomolochus bellones*. The prevalence and intensity reach 34.88% and 1.6 respectively (Table 2; Figs 1G and 2A). All specimens were females and reproductively active by possessing egg sacs or maturing ovaries. The inner side of the operculum forms the specific site for the attachment of this species.

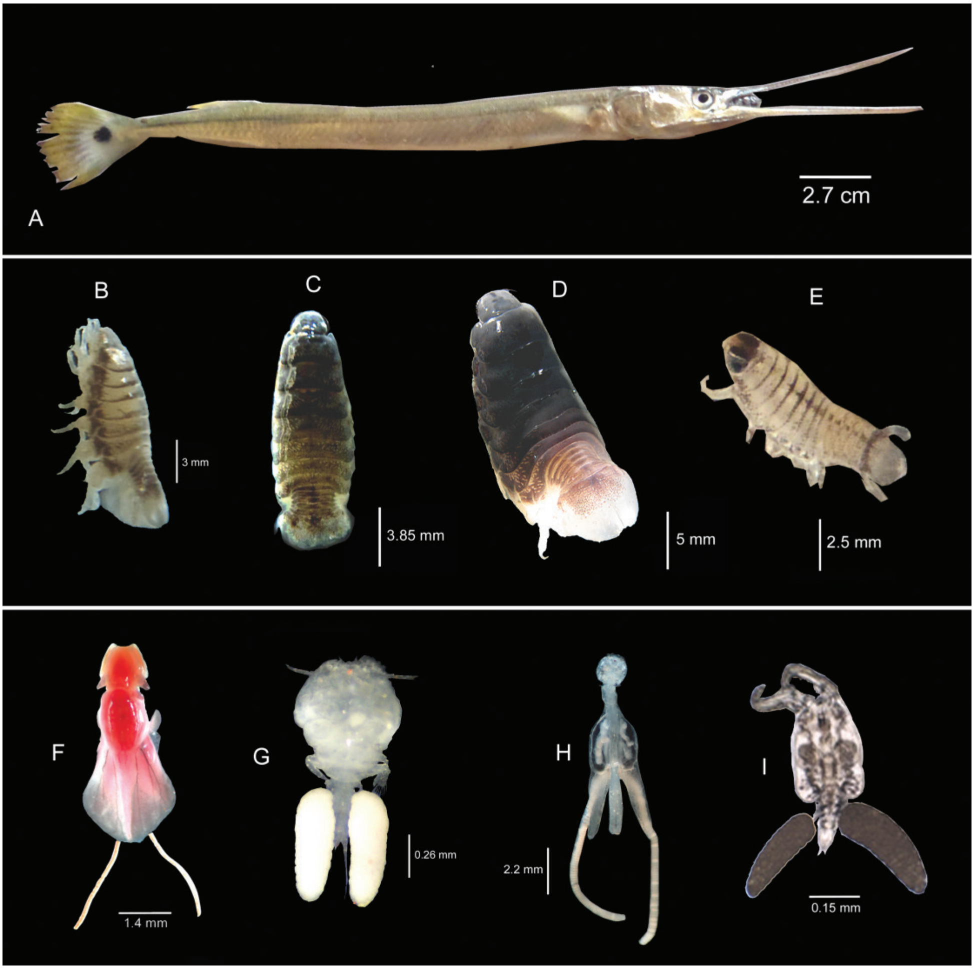

Figure 1. A Host fish *Strongylura strongylura* **B–E** *Cymothoa frontalis* **B** male **C** transitional **D** female **E** juvenile **F** *Lernanthropus tylosuri* – female **G** *Bomolochus bellones* – female **H** *Caligodes lacinatus* – female **I** *Dermoergasilus coleus.*

Dermoergasilus coleus showed the lowest prevalence (23.26%), only 10 of the 43 *S. strongylura* examined being infested; 32 parasites were recovered from the gill filament of infested host fishes, the intensity being equal to 3.2 (Table 2; Figs1I and 2 A). All females were reproductively active possessing growing ovaries and/or egg sacs. *D. coleus* also showed strict site specificity by infesting only the gill filament of the fish. The infestation of *D. coleus* is found to be restricted to certain months (August, October and December–March) only.

Interestingly, the host fish (*S. strongylura*) was under frequent and simultaneous multi infestation (quadruple/triple/double) by any four/three/two of these five parasitic crustaceans (*C. frontalis*, *L. tylosuri*, *C. lacinatus*, *B. bellones* and *D. coleus*) throughout the study period (April 2011 to March 2012) (Table 1; Fig 2B–E).

Table 1. Multiple parasitism by crustaceans on *Strongylura strongylura*.

Month	Host fish series	Number of Parasitic Crustaceans										Remarks
		Isopod		Copepods								
		Cymothoa frontalis		*Lernanthropus tylosuri*		*Caligodes lacinatus*		*Bomolochus bellones*		*Dermoergasilus coleus*		
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
April 2011	1	1	1	12	3	1	-	-	-	-	-	Triple
	2	1	1	7	1	-	-	2	-	-	-	Triple
	3	1	1	-	-	-	-	1	-	-	-	Double
	4	1	-	4	1	-	-	-	-	-	-	Double
	5	-	-	4	-	2	-	-	-	-	-	Double
May 2011	6	-	-	8	-	-	-	1	-	-	-	Double
	7	1	-	4	-	-	-	-	-	-	-	Double
	8	1**	1**	-	-	2	-	-	-	-	-	Double
June 2011	9	1	1	11	2	2	-	-	-	-	-	Triple
	10	-	-	4	-	-	-	-	-	-	-	Single
	11	1	1	-	-	-	-	2	-	-	-	Double
	12	1	-	3	-	-	-	-	-	-	-	Double
July 2011	13	1**	1**	9	9	2	-	-	-	-	-	Triple
	14	1	1	-	-	-	-	1	-	-	-	Double
	15	-	-	3	3	-	-	-	-	-	-	Single
Aug 2011	16	1*	1	-	-	2	-	2	-	4	-	Quadruple
	17	1	1	5	1	-	-	-	-	-	-	Double
	18	-	-	10	-	1	-	-	-	-	-	Double
	19	1	-	8	-	-		1		4		Quadruple
	20	-	-	6	1	2	-	-	-	-	-	Double
	21	1	1	4	-	-	-	-	-	-	-	Double
September 2011	22	1	1	9	2	-	-	2	-	-	-	Triple
	23	1*	1	3	-	2	-	-	-	-	-	Triple
October 2011	24	-	-	9	-	1	-	-	-	3	-	Triple
	25	1	1	7	-	-	-	-	-	3	-	Triple
	26	1	1	2	1	-	-	1	-	-	-	Triple
November 2011	27	-	-	11	2	2	-	-	-	-	-	Double
	28	1	-	2	-	-	-	2	-	-	-	Triple
	29	1	1	6	-	-	-	-	-	-	-	Double
December 2011	30	1	1	7	-	2	-	-	-	-	-	Triple
	31	1	-	3	-	-	-	2	-	-	-	Triple
	32	1**	1**	8	2	2	-	-	-	-	-	Triple
	33	1	1	-	-	2	-	1	-	3	-	Quadruple
January 2012	34	1	1	-	-	2	-	-	-	-	-	Double
	35	1*	-	7	2	-	-	-	-	3	-	Triple
	36	-	-	5	-	-	-	2	-	-	-	Double
February 2012	37	-	-	4	-	2	-	-	-	-	-	Double
	38	1	1	12	4	-	-	-	-	2	-	Triple
	39	1*	1	-	-	-	-	-	2	-	-	Double
	40	1	1	3	-	-	-	-	-	4	-	Triple

Month	Host fish series	Number of Parasitic Crustaceans										Remarks
		Isopod		Copepods								
		Cymothoa frontalis		*Lernanthropus tylosuri*		*Caligodes lacinatus*		*Bomolochus bellones*		*Dermoergasilus coleus*		
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
March 2012	41	-	-	9	-	2	-	-	-	-	-	Double
	42	1**	1**	5	-	-	-	2	-	4	-	Quadruple
	43	1	1	4	-	-	-	-	-	2	-	Triple
	43	24 ** - 4 * - 4	21 ** - 4	218	34	29	0	23	0	29	0	Single – **2** Double – **20** Triple – **17** Multiple – **4**
		32(57) * -Transitional stage = 4 ** - Juvenile = 8		35(252)		17(31)		15(24)		10(32)		

Table 2. Parasitological index of the parasitic crustaceans on *Strongylura strongylura* under multiple parasitism.

Parasites	Prevalence and Intensity	Site of infestation
Cymothoa frontalis Milne Edward, 1840	74.42; 1.78	floor of the buccal cavity
Lernanthropus tylosuri Richard, 1880	81.4; 7.2	on the gill filament
Caligodes lacinatus Kroyer, 1863	39.53; 1.82	penetrating the fleshy part of the lower beak
Bomolochus bellones Burmeister 1835	34.88; 1.6	attached on the inner side of the operculum
Dermoergasilus coleus (Cressey in Cressey & Collette, 1970)	23.26; 3.2	on the gill filament

Quadruple parasitism

Approximately 9% of the observed (4 out of 43) fishes showed the presence of quadruple parasitism, being simultaneously infested by any of the four species of parasitic crustaceans in two different combinations (1. *C. frontalis*, *L. tylosuri*, *B. bellones* and *D. coleus* (CLBD) (50%; 2 out of 4) and 2. *C. frontalis*, *C. lacinatus*, *B. bellones* and *D. coleus* (CC*l*BD) (50%; 2 out of 4)) only during the months of August, December and March (Tables 1 and 3; Fig. 2B and E).

Triple parasitism

Seventeen (out of 43; 39.53%) members of *S. strongylura* showed triple parasitism by simultaneous infestation by any of the three parasitic crustacean species in following four possible combinations: 1) *C. frontalis*, *L. tylosuri* and *C. lacinatus* (CLC*l*), 2) *C. frontalis*, *L. tylosuri* and *B. bellones* (CLB), 3) *C. frontalis*, *L. tylosuri* and *D. coleus* (CLD) and 4) *L. tylosuri*, *C. lacinatus* and *D. coleus* (LC*l*D). The rates of these combinations were

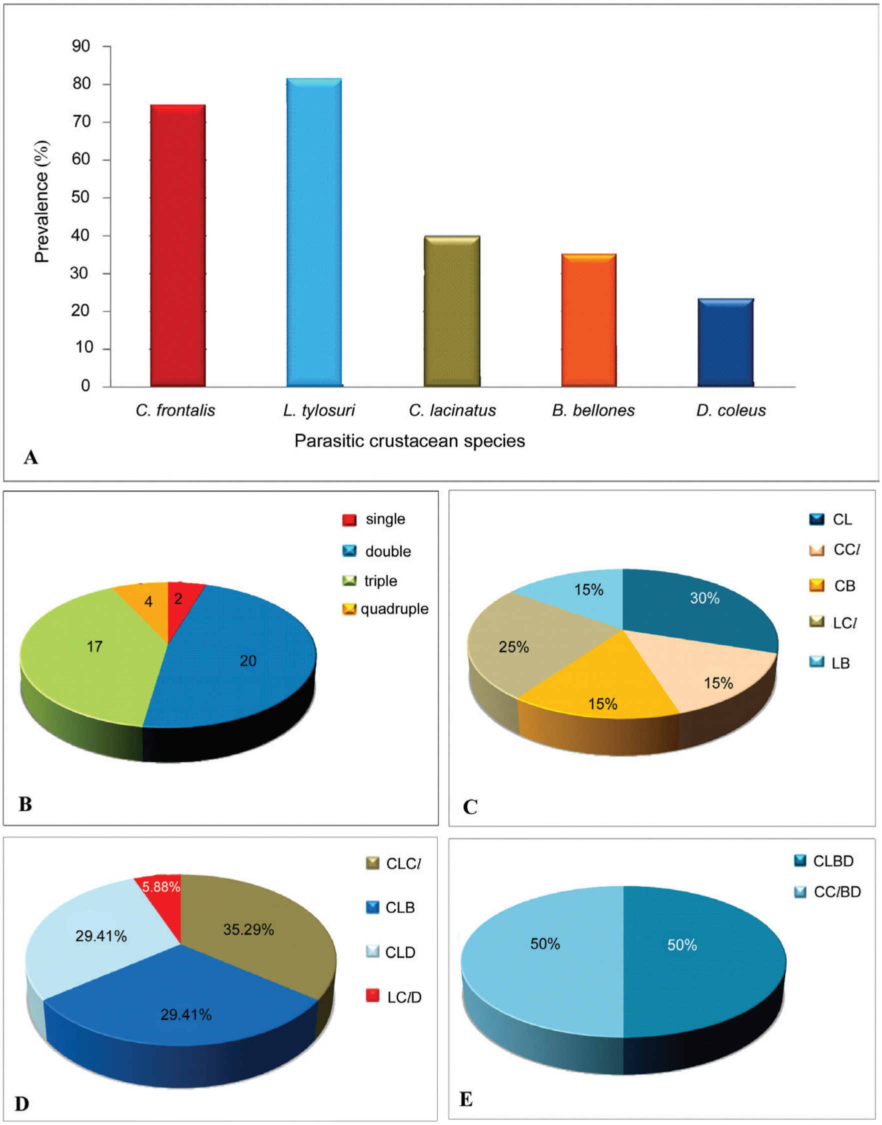

Figure 2. A Simultaneous occurrence of parasitic crustaceans (*Cymothoa frontalis*, *Lernanthropus tylosuri*, *Caligodes lacinatus* and *Bomolochus bellones*, *Dermoergasilus coleus*) parasitizing the fish *Strongylura strongylura* **B** Levels of single, double, triple and quadruple crustacean parasitism on the fish *S. strongylura* **C** Double parasitism on the fish *S. strongylura* – different combinations is represented in percentage **D** Triple parasitism on the fish *S. strongylura* – different combinations is represented in percentage **E** Quadruple parasitism on the fish *S. strongylura* – different combinations is represented in percentage. Legends: CL – *Cymothoa frontalis* and *L. tylosuri* ; CC*l* – *Cymothoa frontalis* and *Caligodes lacinatus*; CB – *Cymothoa frontalis* and *B. bellones*; LC*l* – *L. tylosuri* and *Caligodes lacinatus*; LB – *L. tylosuri* and *B. bellones*; CLC*l* – *Cymothoa frontalis*, *L. tylosuri* and *Caligodes lacinatus*; CLB – *Cymothoa frontalis*, *L. tylosuri* and *B. bellones*; CLD – *Cymothoa frontalis*, *L. tylosuri* and *D. coleus*; LC*l*D – *L. tylosuri*, *Caligodes lacinatus* and *D. coleus*; CLBD – *Cymothoa frontalis*, *L. tylosuri*, *B. bellones* and *D. coleus*; CC*l*BD – *Cymothoa frontalis*, *Caligodes lacinatus*, *B. bellones* and *D. coleus*.

Table 3. Different parasitic combinations of multiple parasitism.

NFO	Single Parasitism	Double Parasitism					Triple Parasitism				Quadruple Parasitism	
	L	CL	CCl	CB	LCl	LB	CLCl	CLB	CLD	LClD	CLBD	CClBD
43	2	6	3	3	5	3	6	5	5	1	2	2
		Total – 20					Total – 17				Total – 4	
	Total :43											

(Legends: CL – *Cymothoa frontalis* and *L. tylosuri* ; CC*l* – *Cymothoa frontalis* and *Caligodes lacinatus*; CB – *Cymothoa frontalis* and *B. bellones*; LC*l* – *L. tylosuri* and *Caligodes lacinatus*; LB – *L. tylosuri* and *B. bellones*; CLC*l* – *Cymothoa frontalis*, *L. tylosuri* and *Caligodes lacinatus*; CLB – *Cymothoa frontalis*, *L. tylosuri* and *B. bellones*; CLD – *Cymothoa frontalis*, *L. tylosuri* and *D. coleus*; LC*l*D – *L. tylosuri*, *Caligodes lacinatus* and *D. coleus*; CLBD – *Cymothoa frontalis*, *L. tylosuri*, *B. bellones* and *D. coleus*; CC*l*BD – *Cymothoa frontalis*, *Caligodes lacinatus*, *B. bellones* and *D. coleus*)

35.29% (CLC*l*), 29.41% (CLB), 29.41% (CLD) and 5.88% (LC*l*D) respectively (Tables 1 and 3; Fig. 2B and D). Instances of triple parasitism were observed throughout the study period except May and August (Table 1).

Double parasitism

The instance of double parasitism in *S. strongylura* was relatively high. Twenty (out of 43; 46.51%) members of the host fish were under simultaneous infestation with any of the two crustacean species. Five possible combinations of double parasitism were detected 1) *C. frontalis* and *L. tylosuri* (CL), 2) *C. frontalis* and *C. lacinatus* (CC*l*) 3) *C. frontalis* and *B. bellones* (CB) 4) *L. tylosuri* and *C. lacinatus* (LC*l*), 5) *L. tylosuri* and *B. bellones* (LB). CL and LC*l* combinations were found to be significantly high amounting 30% and 25% respectively. The percentage of CC*l*, CB and LB combinations were found to be equal (15% each) (Table 3; Figs 2B and C). No incidence of double parasitism was noticed in September, October and December (Table 1).

Single parasitism

Unlike triple and double parasitism noticed in the studied host fish *S. strongylura*, infestation with only one species of parasitic crustacean (single parasitism) was uncommon during the entire study period; only two fishes (out of 43; 4.65%) showed single parasitism with *L. tylosuri*, one in June and other in July (Tables 1 and 3; Fig. 2B).

Discussion

The present study revealed that the spot tail needle fish, *Strongylura strongylura* is a potential host for accommodating five parasitic crustacean species. 396 parasitic crustaceans including 57 cymothoid isopod (*Cymothoa frontalis*) and 339 copepods (252 *Lernanthropus tylosuri*, 31 *Caligodes lacinatus*, 24 *Bomolochus bellones* and 32 *Dermoergasilus coleus*) were recovered from 43 examined fish, *S. strongylura*. The highest prevalence (P = 81.4%) was exhibited by the copepod, *L. tylosuri* throughout the study period. The parasitic cymothoid, *C. frontalis* was recovered from 32 (P = 74.42%) host fishes (*S. strongylura*). The parasitic copepod, *C. lacinatus* was collected from 17 host fishes, its prevalence being 39.53%. The prevalence of *B. bellones*, infesting only 15 of 43 examined fishes, being 34.88 %. *D. coleus* parasitizing 10 *S. strongylura* exhibited lowest prevalence (23.26%). The mean intensity vary according to the parasitic species. The highest intensity was observed in *L. tylosuri* (I = 7.2), the second highest intensity was exhibited by *D. coleus* (I = 3.2). *C. lacinatus* and *C. frontalis* have an intensity reaching 1.82 and 1.78 respectively. The lowest intensity was observed in *B. bellones* (1.6). Among the four copepod species recovered during the present study, the species such as *L. tylosuri*, *C. lacinatus* and *B. bellones* were also reported to be the members in simultaneous multiple infestation on *S. leiura* (Aneesh et al. 2013b). But the intensity and prevalence of *L. tylosuri* is found to be higher in *S. strongylura* than *S. leiura*, suggesting that *S. strongylura* is a more suitable host fish for *L. tylosuri*. The prevalence of *C. lacinatus*, on the other hand, found to be very low in the present host (*S. strongylura*). The prevalence and intensity of *B. bellones* was found to be more or less equal in both fishes, *S. strongylura* (present study) and *S. leiura* (Aneesh et al. 2013b).

Interestingly, these five parasitic crustaceans showed site specific attachment, apparently for avoiding the inter-parasitic competition for space and food. The blood feeding parasite, *C. frontalis*, prefers floor of the buccal cavity and the copepods, *L. tylosuri* and *D. coleus*, prefer the gill filament for their infestation. On the other hand, the tissue feeding, *C. lacinatus* penetrates the tissue lining of the lower beak and *B. bellones* clings the operculum. The site specific attachment of parasitic crustaceans involved in the simultaneous infestation was also reported in the previous study on *S. leiura* from which *L. tylosuri*, *C. lacinatus* and *B. bellones* were recovered from the gill filament, the tissue lining of the lower beak and the operculum respectively of the host fish (*S. leiura*) (Aneesh et al. 2013b), suggesting that the site of attachment of parasitic copepods is highly specific even though their hosts are different.

In the present study, all the collected parasitic copepods belonging to the species, *C. lacinatus* (31) and *B. bellones* (24) and *D. coleus* (32), were invariably matured females carrying egg sac. No single instance of parasitization was noticed by male members of these copepod species (*C. lacinatus*, *B. bellones* and *D. coleus*) apparently due to the existence of sex specific parasitization. Like in *S. leiura*, 34 out of 252 members of recovered copepod species of *L. tylosuri* were males, clinging the genital segments of their parasitic females, apparently in copulatory position. Reports show that only reproductively mature female copepods are parasitic and the males dye after copulation (Jithendran et al. 2008).

Despite the few reports on double parasitism, our knowledge on the occurrence of simultaneous multiple parasitism involving crustaceans is very poor. The occurrence of double parasitism with a copepod, *Pennella* sp. and a cirriped, *C. virgatum* was reported from the flying fish, *C. speculiger* (Daniel and Premkumar 1967). The simultaneous infestation of *N. phaiopleura* and the copepod, *L. sprattae* on the engraulidaen fish *S. commersonii* was reported from Parangipettai (India) (Rajkumar et al. 2006). Double parasitism with the isopod, *M. plagulophora* and the copepod, *L. hemiramphi* was noticed in *Hemiramphus far* (Gopalakrishnan et al. 2010). Daniel and Rao (1967) reported the parasitization of the flying fish by isopod, copepod and cirriped. Recently from our laboratory, the occurrence of double, triple and quadruple parasitism with an isopod *Mothocya renardi* and three copepods such as *L. tylosuri*, *C. lacinatus* and *B. bellones* was reported in the banded needle fish. In the present study, 46.51% (20 out of 43) members of fish, *S. strongylura* showed the occurrence of double parasitism involving one isopod and four copepods in five different combinations. The degree of the occurrence of the combinations *C. frontalis* and *L. tylosuri* (CL) (30%; 6 out of 20), *L. tylosuri* and *C. lacinatus* (LC*l*) (25 %; 5 out of 20) and remaining three combinations , *C. frontalis* and *C. lacinatus* (CC*l*), *C. frontalis* and *B. bellones* (CB) and *L. tylosuri* and *B. bellones* (LB) 15 % each. In three combinations (CL, LC*l* and LB), the copepod, *L. tylosuri* was found to be common; similarly the isopod, *C. frontalis* was the common member in the combinations such as CL, CC*l* and CB, signifying its high rate of infestation on the host. In two double parasitic combinations (LC*l* and LB), all members are copepods.

Apart from previous report from our laboratory, no further information is available on triple parasitism by crustaceans. The present study revealed that 17 members (39.5%) of *S. strongylura* had been under triple parasitism with crustacean species. Out of four combinations (CLC*l*, CLB, CLD and LC*l*D) noticed in the triple parasitism, CLC*l* (*C. frontalis*, *L. tylosuri* and *C. lacinatus*) scored the highest percentage (35.29) (Tables 1 and 3). Interestingly, the existence of quadruple parasitism being simultaneously infested by any of the four species of parasitic crustaceans in two different combinations on the fish, *S. strongylura* was also exposed through the present study. However, its frequency was relatively less (9%) occurring only during the months of August, December and March (Tables 1 and 3; Figs2 B and E). The isopod, *C. frontalis* and copepods, *B. bellones* and *D. coleus* are found to be the common members in two combinations.

Interestingly, *L. tylosuri* appears as a common parasitic crustacean species infesting *S. strongylura* irrespective of the type of parasitism (single, double, triple and quadruple parasitism) involved.

Parasitic crustaceans have negative impacts on their host fishes; their attachment and feeding activities are responsible for any primary diseases that develop due to parasitization (Bharadhirajan et al. 2013). As previously reported in *S. leiura*, the present study, also helped us to identify severe damages induced to the floor of the buccal cavity, the gill filament, the fleshy part of the lower beak, and the inner side of the operculum of the host (*S. strongylura*). Reports showed that, the infestation by parasitic copepods and isopods induce bacterial and viral diseases in parasitized fishes

(Nigrelli 1950, Cusack and Cone 1985, Simudu and Tsummoto 1985, Ravichandran et al. 2001, Ravichandran and Ajithkumar 2008). Recent study showed that, the host (*S. leptolepis*) tissues infected by *N. depressa* were vigorous with disrupted epidermis, damaged muscle fibers and demised collagen matrix; at the pereopod attachment sites, healthy tissues were absent and infested tissues appear to be deteriorated (Rameshkumar and Ravichandran 2013).

In conclusion, the spot-tail needlefish, *Strongylura strongylura* is a potential host for five parasitic crustacean species which showed site specific attachment, may be for avoiding the inter-parasitic competition for space and food. No single instance of parasitization was noticed by male members of these copepod species signifying female specific parasitization. The frequent occurrence of double and triple parasitism and few instance of quadruple parasitism (at the ratio 5:4:1) by parasitic crustaceans noticed on the fish *S. strongylura* indicate that it is not an accidental incident. The multi infestation observed in the present study probably leads the high levels of secondary infections and more studies on this aspect is highly warranted.

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RESEARCH ARTICLE

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Susceptibility to an inoculum of infectious hypodermal and haematopoietic necrosis virus (IHHNV) in three batches of whiteleg shrimp *Litopenaeus vannamei* (Boone, 1931)

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Abstract

The present study evaluated the susceptibility of three different batches of whiteleg shrimp *Litopenaeus vannamei* from Mexico to an inoculum of infectious hypodermal and haematopoietic necrosis virus (IHHNV). Each of the three shrimp batches came from a different hatchery. Because of their origin, it was possible that the genetic makeup of these batches was different among each other. The three batches tested showed differences in IHHNV susceptibility. Here, susceptibility is defined as the capacity of the host to become infected, and it can be measured by the infectivity titer. Susceptibility to IHHNV was observed in decreasing order in shrimp from batch 1 (hatchery from El Rosario, Sinaloa), batch 3 (hatchery from Nayarit) and batch 2 (hatchery from El Walamo, Sinaloa), respectively. The largest susceptibility difference between batches was 5012 times, and that between early and late juveniles from the same batch was 25 times. These results indicate that within a species, susceptibility to a pathogen such as IHHNV can have large differences. Susceptibility to pathogens is an important trait to consider before performing studies on pathogenesis. It may influence virological parameters such as speed of replication, pathogenicity and virus titer. In order to evaluate the potential use of IHHNV as a natural control agent against white spot syndrome virus (WSSV), it is necessary to know host susceptibility and the kinetics of IHHNV infection. These features can help to determine the conditions in which IHHNV could be used as antagonist in a WSSV infection.

Keywords

Litopenaeus vannamei, shrimp batches, shrimp susceptibility, IHHNV, infectivity titer, pathology

Introduction

Infectious hypodermal and haematopoietic necrosis virus (IHHNV) was first detected in stocks of the blue shrimp *Litopenaeus stylirostris* in Hawaii in 1980, where it caused high mortalities (Lightner et al. 1983). Later, it was observed that IHHNV induced runt deformity syndrome (RDS) in infected whiteleg shrimp *L. vannamei* (see Kalagayan et al. 1991). In Mexico, mortalities caused by IHHNV were so severe that farmers were forced to switch from the widely cultured blue shrimp to the less cultured whiteleg shrimp. The latter species soon became the main cultured species since the late 1980s (Lotz 1997). This virus has affected other penaeid species in the Pacific (Alcivar-Warren et al. 1997) and various countries in America, Asia and Oceania (Lightner 1996).

Records from the Gulf of California indicate that IHHNV appeared later than 1987 (see Morales-Covarrubias et al. 1999a). In 1990, the virus was present in hatcheries and farms from Sinaloa and Sonora, where the two shrimp species (whiteleg or blue) were grown. The same year, IHHNV prevalence in wild populations of *L. vannamei*, *L. stylirostris* and *Farfantepenaeus californiensis* from the Gulf of California varied from 46% to 26% (Pantoja et al. 1999). In 1996, IHHNV prevalence in the Platanitos area in Nayarit was up to 86–89% in wild adult *L. stylirostris* females (Morales-Covarrubias et al. 1999b). In the year 2000, a survey carried out off the coast of Panama in wild *L. vannamei* broodstock showed that IHHNV prevalence was 28% (Nunan et al. 2001). Other locations where IHHNV has been reported include Colombia (1990), Texas (1991), Ecuador (1992), Panama (1997) (see Tang and Lightner 2002), Australia (1992, 1993) (see Owens et al. 1992, Krabsetsve et al. 2004); Philippines (1996), Thailand (2000), Taiwan (2001), Tanzania (2000), Madagascar (2000), Mauritius (2000) (see Tang et al. 2003a), Argentina (2003–2007) (see Martorelli et al. 2010) and China (2007) (see Yang et al. 2007). Shrimp species reported to be affected by IHHNV are: farmed and wild specimens of *L. vannamei*, *L. stylirostris*, *Penaeus monodon*, *P. semisulcatus*, wild *Artemesia longinaris*, *F. californiensis*, and hybrid *Penaeus monodon* × *P. esculentus* (Escobedo-Bonilla 2013).

The IHHNV belongs to the family Parvoviridae (Bonami et al. 1990). It has an icosahedral shape, 22 nm in diameter with a single-stranded DNA molecule. Its genome is 4075 nucleotides long (Shike et al. 2000, Tang and Lightner 2006, Robles-Sikisaka et al. 2010), which makes it one of the smallest genomes known for viruses. Since the spread of this virus to other countries, its prevalence in farmed populations of *L. vannamei* has remained steady without obvious clinical signs of disease (Kalagayan et al. 1991).

Currently, IHHNV does not cause high mortalities in farmed populations of blue (*L. stylirostris*) or white (*L. vannamei*) shrimp. Conversely, recent reports indicate that IHHNV infection may protect shrimp against a subsequent white spot syndrome virus (WSSV) infection reducing mortality (Tang et al. 2003b, Bonnichon et al. 2006, Melena et al. 2006). At present, WSSV is the most lethal pathogen to cultured shrimp since its discovery in 1992 (Escobedo-Bonilla et al. 2008, Lightner 2011).

Despite the fact that IHHNV has been present in shrimp farming for over 30 years, little is known about certain features, such as shrimp susceptibility to the virus and speed of virus replication. Here, susceptibility is defined as the capacity of the host to become infected, and it can be measured by the infectivity titer (White and Fenner 1994). It is important to know these IHHNV traits in order to determine whether this virus could be used as antagonist in a WSSV infection using known infectious doses.

Studies carried out with IHHNV compared shrimp susceptibility between species such as the *L. vannamei* and the black tiger shrimp *Penaeus monodon* in Thailand. Methods used to determine IHHNV infection were histology, *in situ* hybridization and transmission electronic microscopy. Chayaburakul et al. (2005) found that *P. monodon* was far less susceptible to IHHNV than the whiteleg shrimp. Likewise, IHHNV susceptibility in the Brazilian species *Farfantepenaeus subtilis* was determined through PCR and bioassays (Coelho et al. 2009). In this species, IHHNV infection was transient for 10 d post inoculation (dpi) when low mortalities were reported (7.5–10%), and a few shrimp (3–7.5%) were IHHNV-positive. After 10 dpi, no IHHNV-infected animals were detected by PCR and no mortalities were recorded (Coelho et al. 2009).

Susceptibility differences to IHHNV and *Baculovirus penaei* were assessed within families of *L. vannamei* (see Alcivar-Warren et al. 1997): crosses of five families of high-health shrimp with high growth rate (family 1.3) or low growth rate (family 1.6) were evaluated. This study found a relationship between levels of genetic diversity determined by restriction analysis polymorphism and IHHNV prevalence. The highest prevalence of IHHNV occurred in the crossing with lower genetic diversity. In contrast, the lowest IHHNV prevalence was observed in crossing with highest genetic diversity (Alcivar-Warren et al. 1997).

The present study aimed to determine under experimental conditions the susceptibility to an IHHNV inoculum in Mexican batches of whiteleg shrimp *L. vannamei* from three different hatcheries.

Methods

Shrimp batches

Three batches of Mexican *L. vannamei* were used to evaluate their susceptibility to IHHNV. Information about these batches, including mean weight, is presented in Table 1. Each batch was transported to the Laboratory of Aquaculture, CIIDIR Unidad Sinaloa where shrimp were placed in 1000 l tanks provided with a recirculation system. Shrimp were acclimated in seawater at a salinity of 25 g/l, temperature ≤ 30 °C and constant areation. Upon arrival to the laboratory, 30 shrimp from each batch were individually screened through PCR analyses to determine its sanitary status. All batches were negative to IHHNV and WSSV.

Table 1. Hatcheries and origin of the three *Litopenaeus vannamei* batches used in the experiments.

Batch	Origin	Collection site	Mean body weight at collection (g)
1	El Rosario, Sinaloa	Granja Aracelitas, Guasave	1.4
2	El Walamo, Sinaloa	El Walamo, Sinaloa	0.7
3	Nayarit, Mexico	Acuícola Machado, Guasave	2.3

Shrimp maintenance and experimental conditions

Animals were fed at a rate of 5% biomass with pelleted feed (Camaronina 30% Purina, Mexico), split into two daily rations (9:00 h in the morning and 17:00 h in the evening). Tanks had a recirculation system, mechanic filtration and continuous areation. Shrimp were maintained under these conditions until used in the susceptibility assays. Experimental conditions during IHHNV challenge assays were: temperature 27 ± 2 °C, salinity 25 g/l and constant areation. Water exchange (70%) was done every week. Shrimp maintenance was assured by feeding each shrimp with three pellets in the morning and three pellets in the evening.

Virus inoculum

An IHHNV inoculum was produced from naturally infected shrimp collected from a farm located in Guasave, Mexico in 2010. The inoculum was produced according to the methods described by Escobedo-Bonilla et al. (2005). The IHHNV-infected shrimp as determined by PCR were used. Tissues from the peraeon without cuticle and hepatopancreas were minced and homogenized in nine volumes of 2X phosphate buffered saline (PBS) (174 mM NaCl, 5.4 mM KCl 2.7, 20 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4). The suspension was clarified by centrifugation (Labnet PrismR, NJ, USA) at 4,000 × g for 10 min at 4 °C and 13,000 × g for 20 min. at 4 °C and filtered through 0.45 µm. The suspension was aliquoted in 1.8 ml cryotubes and stored at -70 °C until used.

In vivo titration

In vivo titration was performed according to the methods described by Escobedo-Bonilla et al. (2005). Five serial dilutions (10^{-1}–10^{-5}) of the IHHNV inoculum were prepared with PBS. Per dilution, five shrimp were individually kept in 19 l plastic tanks containing 12 l seawater (salinity 25 g/l, temperature 27 ± 2 °C). Each dilution was intramuscularly inoculated (100 µl) to the respective shrimp. Animals were followed for 20 d to determine the time at which they became infected. To do this, a pleopod was collected at 5, 10, 15 and 20 dpi. Total DNA was extracted and used for PCR analyses. Infection data were used to determine the shrimp infectious titer 50% endpoint (SID_{50}/ml) according to the Reed and Muench method (Reed and Muench 1938).

DNA extraction

DNA extraction was carried out with pleopod tissues using DNAzol (MRC Cincinnati, OH, USA) following manufacturer's instructions. Individual pleopods were homogenized in 1.5 ml Eppendorf tubes containing 500 µl DNAzol, incubated 10 min at room temperature, centrifuged at 13,000 × g for 10 min. DNA was precipitated with absolute ethanol mixing by inversion and incubating at room temperature for 3 min and centrifuged at 7,500 × g for 5 min. The pellet was washed twice with 75% ethanol and centrifuged at 13,000 × g for 2 min. The pellet was air-dried and resuspended in 30 µl ultrapure water (Life Technologies, USA).

PCR analyses

The IHHNV and/or WSSV PCR analyses were done in 200 µl microtubes. Each microtube contained 24 µl of the PCR mix [18.8 µl ultrapure water, 2.5 µl 10X PCR buffer (Biolase, Irvine CA, USA), 1.0 µl $MgCl_2$ (50 mM Biolase, USA), 0.5 µl dNTPs (10 mM, Biolase, USA), 0.5 µl of each primer: IHHNV392F (5'-gggCgAACCAgAATCACTTA-3'), IHHNV392R (5' ATCCggAggAATCTgATgTg 3') (Tang et al. 2000), or in-house designed WSSV *vp28* gene primer pair: VP28F1 (5' CTCTTTCggTCgTgTCggCC 3') and VP28R2 (5'gAgACgggggTgAAggAggAgg 3') (Escobedo-Bonilla, unpubl.), and 0.2 µl Taq DNA polymerase]. Each tube contained 1.0 µl DNA from each of the samples as template. Total reaction volume was 25 µl. For both viruses, PCR was performed under the following conditions: initial denaturation 95 °C for 4 min. Then, 35 cycles with the following: denaturation 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 5 min. The reaction was stopped at 4 °C. The PCR product for IHHNV was a band of size of 392 bp, whereas the PCR product for WSSV was 300 bp.

Evaluation of IHHNV susceptibility

PCR infection data was used to determine the infectivity titer (SID_{50}/ml) for each shrimp batch. Susceptibility of shrimp batches to IHHNV was determined with their respective infectivity titers in the different shrimp sizes.

Results

By intramuscular inoculation, 15 d were required to determine IHHNV infectivity titer in susceptible shrimp. Juvenile shrimp (≤ 4.0 g) from Batch 1 had an infectivity titer of $10^{5.2}$ SID_{50}/ml, whereas late juveniles (≥ 8.0 g) had an infectivity titer of $10^{4.6}$ SID_{50}/ml

Table 2. The IHHNV infectivity titers in *Litopenaeus vannamei* Batch 1.

Mean Weight (g)	Dilution	Inoculated shrimp	Infected shrimp	Infectivity titer
3.2 ± 0.78	10^{-1}	5	5	$10^{5.2}$ SID$_{50}$/ml
	10^{-2}	5	5	
	10^{-3}	5	4	
	10^{-4}	5	4	
	10^{-5}	5	0	
10.5 ± 0.85	10^{-1}	5	5	$10^{4.6}$ SID$_{50}$/ml
	10^{-2}	4	4	
	10^{-3}	5	4	
	10^{-4}	5	0	
	10^{-5}	5	0	

Table 3. The IHHNV infectivity titers in *Litopenaeus vannamei* Batch 2.

Mean Weight (g)	Dilution	Inoculated shrimp	Infected shrimp	Infectivity titer
	10^{0}	5	5	
2.9 ± 0.48	10^{-1}	5	0	< $10^{1.5}$ SID$_{50}$/ml
	10^{-2}	5	0	
	10^{-3}	5	0	
	10^{-4}	5	0	
	10^{-5}	5	0	
	10^{0}	5	5	
8.9 ± 0.76	10^{-1}	5	0	< $10^{1.5}$ SID$_{50}$/ml
	10^{-2}	4	0	
	10^{-3}	5	0	
	10^{-4}	5	0	
	10^{-5}	5	0	

Table 4. The IHHNV infectivity titers in *Litopenaeus vannamei* Batch 3.

Size (g)	Dilution	Inoculated shrimp	Infected shrimp	Infectivity titer
	10^{-1}	5	5	
	10^{-2}	5	5	
3.1 ± 0.59 g	10^{-3}	5	1	$10^{3.6}$ SID$_{50}$/ml
	10^{-4}	5	0	
	10^{-5}	5	0	
	10^{-1}	5	3	
	10^{-2}	5	0	
	10^{-3}	5	0	$10^{2.2}$ SID$_{50}$/ml
7.9 ± 0.33 g	10^{-4}	5	0	
	10^{-5}	5	0	

(Table 2). In Batch 2, IHHNV infectivity titer was < $10^{1.5}$ SID$_{50}$/ml since none of the early (≤ 4 g) or late (≥ 8 g) juvenile shrimp inoculated with IHHNV dilutions became infected (Table 3). Shrimp from Batch 3 had an infectivity titer of $10^{3.6}$ SID$_{50}$/ml in

Table 5. Susceptibility differences to IHHNV in *Litopenaeus vannamei* batches.

Batch combination	Same batch early vs. late juveniles	Between batches early juveniles	Between batches late juveniles
1	4 times		
2	----		
3	25 times		
1 vs. 2		5,012 times	1,259 times
1 vs. 3		40 times	251 times
2 vs. 3		126 times	5 times

early juveniles (≤ 4g) and $10^{2.2}$ SID_{50}/ml in late juveniles (≥ 8.0 g) (Table 4). Highest susceptibility to IHHNV infection was recorded in Batch 1, followed by Batch 3, and Batch 2 with the lowest susceptibility (Table 5).

Discussion

The three batches used in the present study came from different brooder stocks located in hatcheries that were at least one-hundred kilometers away from each other. Moreover, most hatcheries have their own brooder stock programs, where they avoid using shrimp from other hatcheries. Therefore, it can be assumed that their genetic makeup is different from each other. The three shrimp batches were maintained under the same controlled environmental conditions (temperature, salinity and areation) and the same IHHNV isolate was used. Therefore, shrimp origin is the variable that explains the susceptibility differences found in this study.

Susceptible shrimp showed higher IHHNV susceptibility at early stages within a single batch (≤ 4.0 g mean body weight) (Tables 2, 4). The relationship between shrimp size and virus susceptibility was first observed in batches of whiteleg shrimp in Hawaii (Kalagayan et al. 1991). A similar finding was also described in blue shrimp: animals of 1 to 5 g were more likely to die due to an IHHNV infection than 14 g juvenile shrimp (Bell and Lightner 1987).

Our results revealed that important intraspecific differences in virus susceptibility might occur. Studying host susceptibility is important since it may influence virological traits such as speed of virus replication, pathogenicity and virulence (White and Fenner 1994). These features determine viral pathogenesis and the outcome of infection. A species with variable susceptibility may produce different responses to a pathogen. Such responses can provide new information on virus pathology and pathogenesis.

A number of factors were mentioned to determine differences in host susceptibility to a pathogen: genetic (presence of defense genes, genetic diversity) (White and Fenner 1994, Alcivar-Warren et al. 1997), physiology (age, size, nutrition, stress) (Lotz 1997), environment (water quality) and pathogen persistence (Flegel 2007). In the present study, shrimp was the only variable in our susceptibility experiments, since virus isolate and environmental factors were the same. Although the genetic diversity of the batches used was not determined, it seems likely that their genetic makeup was different to each other. Thus, differences in IHHNV susceptibility found here may suggest that an intrinsic factor (i.e. genetic diversity) was the cause for such differences. These results agree with another study done with whiteleg shrimp, which indicated that the more diverse the genetic makeup of a shrimp family, the lower the susceptibility to a pathogen (Alcivar-Warren et al. 1997).

A previous work with an IHHNV-resistant selected line of *L. stylirostris* also showed the effect of the host genetic changes in virus tolerance (Tang et al. 2000). When this “resistant” shrimp line was co-cultured with virus-naïve *L. stylirostris*, high IHHNV-related mortalities were recorded (Tang et al. 2000). This finding showed that the virus was fully pathogenic and was carried by the “resistant” shrimp, which were rather tolerant to the virus. Such a tolerance most likely was due to a genetic change in the host and led to the following updated viral accommodation hypothesis (Flegel 2007): “crustaceans and other arthropods actively accommodate viral pathogens as persistent infections that act as a kind of memory that functions to specifically reduce the severity of disease and to dampen viral triggered apoptosis”.

In the present study, shrimp batches with the lowest IHHNV susceptibility always were IHHNV-negative by PCR. This result indicates that a persistent infection (or viral accommodation) was not the cause for reduced IHHNV susceptibility. Instead, it suggests that a genetic or physiological factor in shrimp batches may be associated to a reduced IHHNV susceptibility.

Our study is considered to be a first step to assess the value of IHHNV as a natural agent against a WSSV infection. Previous studies (Tang et al. 2003b, Bonnichon et al. 2006, Melena et al. 2006) revealed that pre-infecting susceptible shrimp with IHHNV reduced infection and mortality due to WSSV. This phenomenon was first described in *L. stylirostris* juveniles (1–3 g mean body weight [MBW]) (Tang et al. 2003b), and later it was also verified with *L. vannamei* larvae (nauplius, zoea 1), postlarvae (pl 22), which also showed a delay in mortality (100% in controls vs. 95% in IHHNV-treated shrimp at 10 d post WSSV challenge) (Melena et al. 2006), and juveniles (3.5 g MBW) (Bonnichon et al. 2006). These studies indicate a possible role of IHHNV as a natural control agent against WSSV infection. The IHHNV causes relatively low damage to farmed shrimp and it may interfere with WSSV replication. In order to assess IHHNV as an antagonist to WSSV, features such as replication speed, pathogenesis and host susceptibility need to be known. This information will help to determine the best conditions at which IHHNV can be more effective against WSSV infection in susceptible shrimp. Shrimp susceptibility to IHHNV is therefore an important trait to consider before performing such studies. A shrimp batch highly susceptible to IHHNV may allow higher virus replication rates before a WSSV challenge. Higher amounts of IHHNV in a susceptible host may impair WSSV replication, thus inducing delayed or reduced mortalities due to a WSSV infection.

As IHHNV infection does not cause mortality to whiteleg shrimp, the present study evaluated shrimp IHHNV susceptibility by their infectivity titers using PCR.

Here, the infectivity titer was a measure of virus susceptibility between shrimp batches. This method may be an indirect way to assess genetic diversity. Further studies are needed to confirm that IHHNV susceptibility may be an indicator of the reduced genetic diversity and/or endogamy of shrimp batches and/or families.

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Preliminary survey of a nemertean crab egg predator, *Carcinonemertes*, on its host crab, *Callinectes arcuatus* (Decapoda, Portunidae) from Golfo de Nicoya, Pacific Costa Rica

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Abstract

The possible presence of egg predators in brood masses of portunid crabs from Pacific Central America has not been studied yet. This survey reports the finding of a nemertean crab egg predator on the portunid crab, *Callinectes arcuatus*, from the Golfo de Nicoya, Pacific Costa Rica. Nemerteans were found in the egg masses of 26 out of the 74 crabs for a prevalence of 35%. The intensity (mean number of worms/ infected crab) was estimated to be 18 with a variance of 1–123 worms/infected crab. No nemerteans were observed either in the 19 *C. arcuatus* from Golfo Dulce (southern Pacific coast) and the 10 *Portunus asper* from Herradura-Jaco (central Pacific coast). This nemertean is a member of the genus *Carcinonemertes*, which has been reported from the Caribbean coast of Panama. However, the encountered *Carcinonemertes* sp. is the first published finding and report from Costa Rica and Pacific Central America.

Keywords

Callinectes arcuatus, egg mortality, crab egg predator, Central America

Introduction

The arched portunid crab, *Callinectes arcuatus* Ordway, 1863 has become an increasingly important growing fishery resource along the Pacific coast from Mexico to Ecuador (Paul 1982, Castro and De Alteris 1989). In the Golfo de Nicoya of the Pacific coast of Costa Rica, Dittel (1993) reported a large population of this portunid crab. More recently, Fischer and Wolff (2006) analyzed the current level of exploitation of this resource by size frequency analysis of trap and trawl catches in the Golfo de Nicoya; the results of this study revealed the potential of increasing catch yield about 20% without a detriment to the crab population. Thus, the *C. arcuatus* fishery is becoming recognized as a commercially important fishery for Costa Rica.

Phylum Nemertea, commonly called ribbon or proboscid worms, comprise approximately 1250 species (Kajihara et al. 2008). These ribbon worms are generally predators using their proboscis to capture prey. One monostiliferous hoplonemertean family, Carcinonemertidae, consists of nemerteans that are ecto-symbiont egg predators of decapods (Wickham and Kuris 1985, 1988, Kuris and Wickham 1987, Kuris 1993, McDermott 2006, Sadeghian and Santos 2010). These worms were implicated to cause 50% brood mortality in the *Cancer magister* fishery in northern California (Wickham 1979).

Carcinonemertids have been reported in Panamanian crabs, but so far only from the Caribbean coast (Collin et al. 2005). As far as we know, no published information is available about the possible infestation of egg masses of portunid crabs along the Pacific coast of Central America. Therefore, this study was conducted to determine if nemertean crab egg predators are present on *Callinectes arcuatus* from Golfo de Nicoya. If these nemerteans are found, then baseline numbers of infestation and incidence can be established to monitor future increases and their impact on a growing fishery.

Methods

The following numbers and species of ovigerous crabs were collected by local fishermen: 74 specimens of *Callinectes arcuatus* from Golfo de Nicoya, central Pacific (10°0'00"N, 85°0'00"W), 19 of *C. arcuatus* from Golfo Dulce, southern Pacific (8°32'16"N, 84°41'35"W), and 10 of *Portunus asper* (A. Milne-Edwards, 1861) from trawls off the coast of Herradura-Jaco, central Pacific (9°64'00"N, 84°65'00"W). All crabs were shipped under ice to the Universidad de Costa Rica in San José and then stored frozen.

After the crabs were allowed to thaw, eight pleopods of each crab were carefully snipped at the bases, wet weighed, and then placed in seawater. A MS5 Leitz dissecting microscope was used to examine the pleopods for nemerteans, worm sheaths, and worm egg strings. Sheaths without worms were considered in the count for infestation. During holding of the crabs in the containers by fishermen, stressed worms have been observed to leave the egg masses, which have become suboptimal for the nemerteans (J. Norenburg, pers. comm.). A total of 824 pleopods were analyzed.

To quantify the worm dispersion and infestation on host crabs, prevalence and intensity were measured. Prevalence, an indicator of infestation, was defined as numbers of infected host crabs per total host crabs × 100. Intensity, indicator of dispersion amongst infected host crabs, was defined as mean number of worms per infected host crab.

Results

Golfo de Nicoya

A total of 26 egg masses out of 74 crabs were found to be infested with worms (Fig. 1A) for a prevalence of 35%. The worm was tentatively identified as belonging to the genus *Carcinonemertes* with a single stylet (Fig 1B). Worm length averaged about ~7.0 mm with widths about 0.13 mm.

Most worms were found to be inside sheaths (Fig. 2a). The mucous sheaths were linear, of small height (15–25 µm), and dome-shaped with uniformly distributed lapillae (Fig 2b). Cursory investigation of the gills of the *C. arcuatus* found no ensheathed nemerteans. Worm egg strings were also observed intertwined around the crab eggs (Fig 3).

Worms were found throughout the egg mass, but more often encountered at the base of pleopods. The pleopodal base of one crab showed pronounced egg mortalities (Fig. 4A) and an accumulation of worms in the mid pleopodal regions (Fig. 4B).

Mean intensity (# worms/infested crab) was 18 (range 1–123 worms). Linear regression analysis showed no significance ($r^2 = 0.12$; correlation coefficient 0.35; $P=0.09$) between crab size (carapace width) and worm intensity (Fig. 5).

Golfo Dulce and Herradura/Jaco

No worm infestations were found on crabs collected from Golfo Dulce and from off the coast of Herradura/Jaco.

Discussion

This survey is the first report of a carcinonemertid crab egg predator on the portunid *Callinectes arcuatus* from Golfo de Nicoya in the Pacific coast of Costa Rica, and as far as we know, also the first published report from the Pacific coast of Central America. The observation of a single stylet near the brain is a characteristic of the genus *Carcinonemertes*. Further histological analysis will be needed to confirm the presence of the Takakura's duct system (Humes 1942). In male *Carcinonemertes*, this duct system includes the vas efferens, vas deferens, seminal vesicle and gonoduct (Shields and Kuris 1990).

Nemerteans have been previously reported on the rafting crab, *Plagusia immaculata* Lamarck, 1818, from Pacific Panamanian coast (M. Torchin, unpubl.) and on

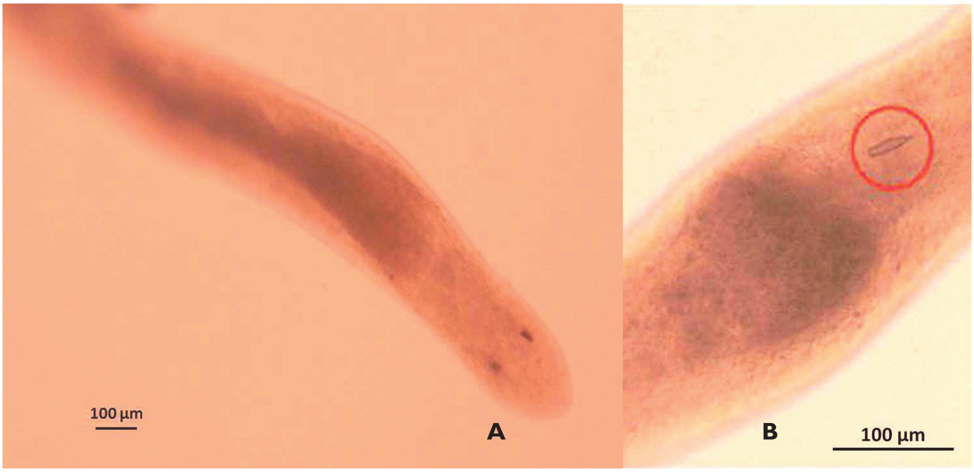

Figure 1. A Unidentified *Carcinonemertes* sp. from an ovigerous female of *Callinectes arcuatus* collected in Golfo de Nicoya, Pacific coast of Costa Rica. **B** Single basis and stylet observed in a nemertean from the egg mass of *Callinectes arcuatus* collected in Golfo de Nicoya, Pacific coast of Costa Rica.

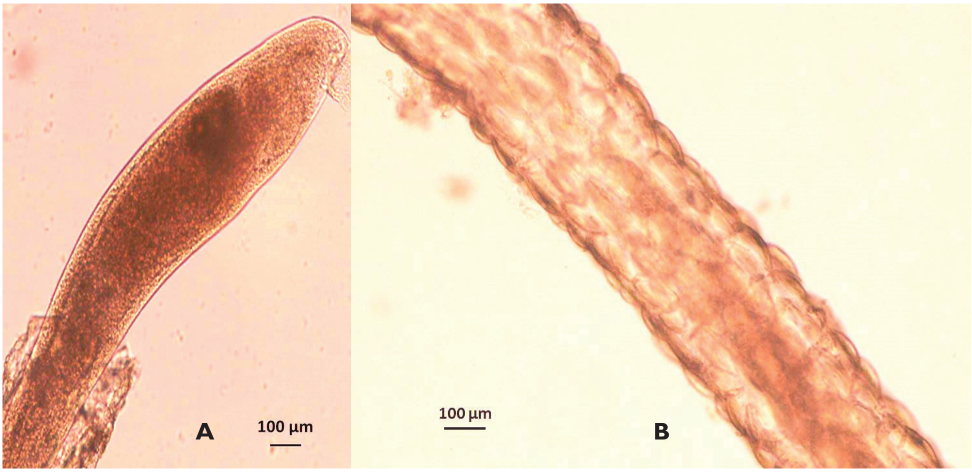

Figure 2. A Nemertean worm emerging from the mucous sheath, observed in the egg mass of *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica. **B** Mucous sheath of a nemertean worm showing pronounced domed lapillae, observed in the egg mass of *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica.

unidentified crabs from Bocas del Toro, Caribbean coast of Panama (Collin et al. 2005). From unpublished 2004 survey of Panamanian crabs from the Caribbean coast (C. Santos, pers. comm.) carcinonemertids have been reported on *Leptodius floridanus* (Gibbes, 1850), *Macrocoeloma trispinosum* (Latreille, 1825), *Mithraculus cinctimanus* (Stimpson, 1860), *Mithraculus forceps* (A. Milne-Edwards, 1875), *Mithrax caribbaeus* (M. J. Rathbun, 1900, *Mithrax spinosissimus* (Lamarck, 1818), *Panopeus lacustris* Desbonne, 1867 and *Pilumnus pannosus* M. J. Rathbun, 1896.

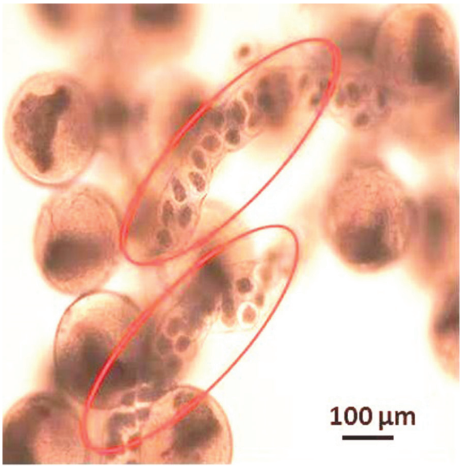

Figure 3. Nemertean worm egg strings (encircled) amongst eggs of *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica.

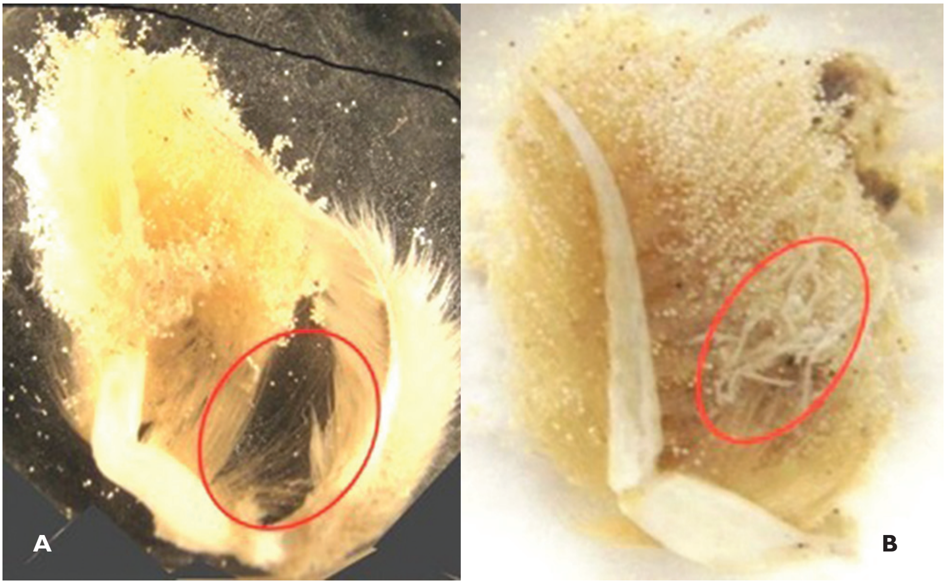

Figure 4. A Severe crab egg mortalities (encircled) at lateral base of a pleopod from an ovigerous female *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica. **B** Nemertean worms (encircled) between the lateral middle and lateral base of a pleopod from an ovigerous female of *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica.

This study found a 35% prevalence of this carcinonemertid on *Callinectes arcuatus* from the population from Golfo de Nicoya and none from Golfo Dulce. Wickham (1979) reported 100% prevalence for *Carcinonemertes errans* on Dungeness crab, *Cancer magister* (Dana, 1852) while Shields et al. (1990) found >97% prevalence

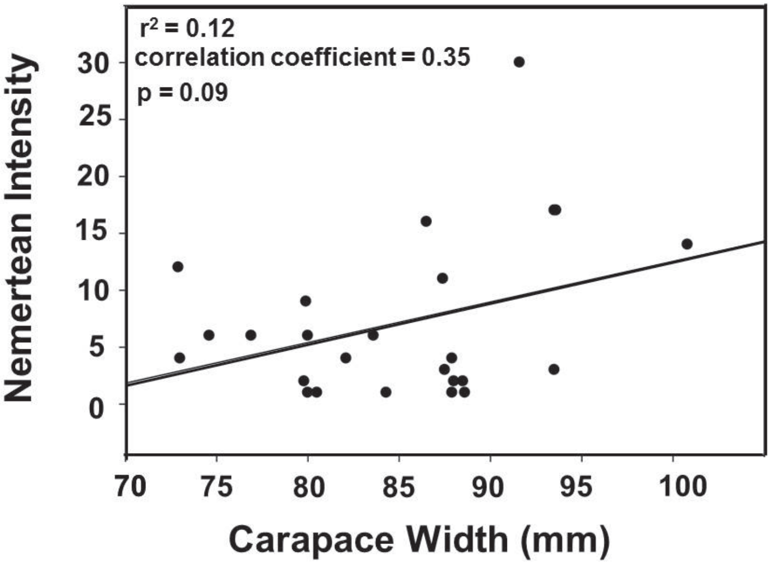

Figure 5. Linear regression analysis between size (carapace width) of ovigerous females of *Callinectes arcuatus*, Golfo de Nicoya, Pacific Costa Rica, and nemertean worm intensity.

on the yellow rock crab, *Cancer anthonyi* (M. J. Rathbun, 1897). Golfo de Nicoya is considered one of the most productive estuaries in the world (Cordoba-Muñoz 1998, Gocke et al. 2001), and is also the most important fishery area in Costa Rica (Cortés and Wehrtmann 2009). The Golfo de Nicoya is highly impacted by human activities and one of the most polluted regions along the Pacific coast of Costa Rica (Vargas 1995). These conditions may enhance the prevalence and transmission of these nemertean crab egg predators. In contrast, Golfo Dulce is a deep tropical, highly stratified and low-productivity fjord (Quesada-Alpízar and Cortés 2006). Especially the limited water circulation in this tropical fjord may restrict dispersion and transmission of nemerteans. Kuris et al. (1991) reported that infestation of nemerteans on the red king crab was highly variable; high egg mortalities were observed in geographically clustered crab populations.

The carcinonemertid encountered in our study may be host-specific to *C. arcuatus* and have not exploited other crab hosts, such as *Portunus asper* from offshore of Herradura and Jaco. Further investigations are needed to determine the extent of nemertean prevalence on other potential host crabs.

In this study, the estimated infestation of 18 is low compared to 46,000 reported for *Carcinonemertes errans* Wickham, 1978 on *Cancer magister* by Wickham (1979) and 692 (86.5 worms/pleopod × 8) for *Cancer anthonyi* (Shields et al. 1990). This low intensity may be a reflection of several factors. Firstly, this symbiotic relationship between the worm and *Callinectes arcuatus* may have evolved most recently. Secondly, the artisan fishery inclusion of small-sized crabs, as well as both males and females, might contribute to the low reproduction of worms, which need ovigerous crabs to complete their reproductive cycle. Therefore, the lower intensities observed in *C. arcuatus* and *C. anthonyi* compared to *C. magister* could be due to the fact that both female and male crabs are taken out in the *C. arcuatus* and *C. anthonyi* fisheries.

Although in this preliminary study, worm incidence was low for *Callinectes arcuatus* from the Golfo de Nicoya, future monitoring of the carcinonemertid is strongly recommended. Collapse of this growing artisan fishery could potentially occur if worm population were somehow to explode, especially in these times of unstable climatic change. In 1981, the red king crab fishery recorded the third highest historical yield; however three years later, the fishery suffered the lowest catch in its 30 year history (Kuris et al. 1991). High red king crab egg mortality was correlated with high-intensity infestations of nemerteans in the egg mass (Kuris et al. 1991).

In the present study, the worms and their sheaths were observed in the medial middle and base areas of the pleopod. Pleopods of one crab sample showed very high egg moralities in these regions (refer to Fig. 4A, B). These findings are in agreement with similar observations from other crabs. Higher worm presence and abundance as well as egg mortalities in these pleopodal areas were also reported for *Cancer anthonyi* (Shields et al. 1990) and for *Cancer magister* (Okazaki and Kuris 2004).

Most spawning of *C. arcuatus* occurs during the dry season from December to April, when ovigerous females migrate from the low salinity areas of the inner gulf to the high salinity waters in the outer gulf where the larvae hatch (DeVries et al. 1983). Larval development is completed within 70 days (Paul 1982, Dittel and Epifano 1984). Further studies are suggested to determine whether nemerteans complete their life cycle on one brood of host eggs or remain on the crab for a subsequent brood to complete another reproductive cycle. After host eclosion, carcinonemertids on portunid crabs encyst between the branchial lamellae and then lie dormant until the female crab oviposits (Humes 1942, Shields and Kuris 1990). In this study examination of branchial chambers of female crabs did not reveal encysted worms but barnacles from the genus *Octolasmis* (unpubl observations).

In this study, frozen crab samples were analyzed due to the logistics and limitations of receiving ovigerous crabs from fishermen. Worms have been observed to leave the crab egg mass when the crabs are out of the water (J. Norenburg, pers. comm.). Future studies using freshly trapped ovigerous crabs are suggested to improve infestation and incidence numbers. Also live nemerteans would allow for further histological and DNA analyses for phylogenetic relationships with other *Carcinonemertes* species.

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