Trends in Terrestrial Isopod Biology

Edited by
Stefano Taiti, Elisabeth Hornung,
Jasna Štrus, Didier Bouchon

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Preface

The present special issue includes papers presented at the 9th International Symposium on Terrestrial Isopod Biology (ISTIB9) held at Poitiers, France (26-30 June, 2014). Terrestrial isopods are fascinating crustaceans that occur in various land habitats from seashores to deserts. Up to date, the group includes more than 3,700 species with a worldwide distribution. They play an important role as decomposers, transforming the organic material into soil, and are important biogeographical indicators due to the limited distribution of many taxa and their low dispersal ability. In recent years, a growing body of work highlights the potential of woodlice as an important regulatory force mediating soil microbial responses to combined global change factors.

This series of symposia on terrestrial isopod biology started in London, UK, in 1983, organized by S.L. Sutton and D.M. Holdich, following a suggestion of M.R. Warburg. Seven symposia followed the first: 1986, Urbino, Italy, organized by R. Argano, P. Del Grande, F. Ferrara, C. Manicastri, H. Schmalfuss and S. Taiti; 1990, Poitiers, France, by J.P. Mocquard and P. Juchault; 1997, Haifa, Israel, by M.R. Warburg and E. Hornung; 2001, Iraklion, Greece, by S. Sfenthourakis; 2004, Aveiro, Portugal, by S. Loureiro and A. Soares; 2007, Tunis, Tunisia, by F. Charfi-Cheikhrouha, M. S. Achouri, S. Hamaïed and K. Nasri-Ammar; and 2011, Bled, Slovenia, by J. Štrus and P. Zidar. In 2014, the 9th meeting was organized again in Poitiers. This rich history provides evidence that the scientific community that has chosen "a somewhat extravagant group of organisms to work with" (as defined by H. Schmalfuss at the symposium in Bled) is very active and dynamic. Seventy-one researchers from 15 countries came to Poitiers to share their common passion for woodlice. The large presence of students (one third of the attendees) represents a great hope for the future. The next symposium is already planned to take place in Budapest, Hungary, in June 2017, organized by E. Hornung and collaborators.

In 2014 the community of terrestrial isopod researchers sadly suffered the loss of an important scientist and our beloved colleague, Michael R. Warburg. The present issue is dedicated to his memory and to his greatest contribution to the knowledge of isopod biology (see commemoration by Hornung).

This volume includes 14 articles out of 36 oral and 28 poster communications presented at ISTIB9, covering topics such as systematics and biogeography, morphology and physiology, evolutionary biology, ecology and ecotoxicology. In particular, new data on taxonomy and biogeographical areas that are still largely unexplored have been presented (see papers by Sfenthourakis & Taiti, Taiti & Wynne, Kashani, and Eshaghi et al.). Morphological and developmental features have also been explored (see papers by Horváthová et al., Csonka et al., Mrak et al., and Giurginca et al.). Population dynamics as well as behaviour related to environmental cues have been studied by

Hornung et al., Dixie et al., and Tuf et al. Finally, new methods facilitating the acquisition of data by using numerical tools and drawing techniques have been developed (see papers by Caubet & Richard, and Montesanto).

Several people and organizations contributed to the success of the Poitiers symposium. We, as members of the Scientific Committee, would like to express our warmest thanks to all of them. We are particularly indebted to colleagues from the laboratory "Ecologie et Biologie des Interactions" (University of Poitiers) who gave their time and enthusiasm for the organization of this symposium. We would also thank here all the colleagues who have helped to revise manuscripts for publication in this special issue of ZooKeys, as well as Dr. Lyubomir Penev and Yordanka Banalieva of Pensoft Publishers who encouraged and supported this publication. Finally, we would like to thank our sponsors, the Région Poitou-Charentes, the University of Poitiers, and the Centre National de la Recherche Scientifique.

Stefano Taiti
Elisabeth Hornung
Jasna Štrus
Didier Bouchon
(Editors)

Conference Photo

9th ISTIB Poitiers 2014

1 Faouzia Charfi **2** Giuseppe Montesanto **3** Mohamed Sghaïer Achouri **4** Lamia Medini-Bouaziz **5** Pierre Grève **6** Didier Bouchon **7** Péter Szabó **8** Myryam Badawi **9** Karima Nasri-Ammar **10** Isabelle Marcadé **11** Hajer Khemaissia **12** Chedliya Ghemari **13** Terézia Horváthová **14** Freddie-Jeanne Richard **15** Isabelle Giraud **16** Jessica Dittmer **17** Margot Fortin **18** Richard Cordaux **19** Karel Tajovský **20** Vladimir Sustr **21** Paula Beatriz Araujo **22** Elisabeth Hornung **23** Diana Csonka **24** Clotilde Hardy **25** Sylvine Durand **26** Christelle Mirebeau **27** Catherine Souty-Grosset **28** Mohamed Amine Chebbi **29** Bernice Dixie **30** Yves Caubet **31** Shigenori Karasawa **32** Rok Kostanjsek **33** Martin Zimmer **34** Pierre Broly **35** Ivan Hadrián Tuf **36** Anas Ayari **37** Maria Kamilari **38** Stefano Taiti **39** Andreas Ziegler **40** Spyros Sfenthourakis **41** Andreas Dimitrou **42** Theodosia Charalambous **43** Lise-Marie Genty **44** Sophie Beltran-Bech **45** Marion Poussin **46** Clément Gilbert **47** Nicolas Bech **48** Emmanouela Karameta **49** Victorien Valette **50** François Lefebvre **51** Frederic Grandjean **52** Mark Hassall.

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In commemoration of Prof. M.R. Warburg and of his contribution to terrestrial Isopod biology (31 May 1931, Berlin–9 February 2014, Haifa)

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Our scientific community has lost one of its prominent members: Prof. Michael R. Warburg (MRW) who passed away on the 9th of February, 2014 in Haifa. The organisers of the 9th Symposium on the Biology of Terrestrial Isopods, held in Poitiers, France, decided to dedicate the meeting and this special issue to his memory. Prof. Warburg was a highly regarded member of our community, a 'spiritual sponsor' of isopod research, a passionate isopodologist himself, and a mentor to many students and young researchers.

M.R. Warburg and the symposia series

The foreword of the 1st Symposium on the Biology of Terrestrial Isopods volume (London, 7–8th July 1983) (Sutton and Holdich 1984) states that MRW was 'the father' of this series of symposia as he originally suggested bringing together "*people with an interest in terrestrial isopods and try to present as wide a range of papers as possible*". The idea proved to be a great success. The first meeting was followed by several others at intervals of 3–5 years. MRW also initiated a meeting in Vancouver, Canada, in 1992.

This symposium, under the umbrella of the American Society for Zoologists' regular congress, was organized by Prof. M.A. Alikhan (Sudbury, Ontario, Canada). MRW also lent his support to a smaller workshop in Hungary (Gödöllő) in 1991 connected to the 4th European Congress of Entomology. E. Hornung and K. Szlavecz organized the workshop, including a round table discussion that resulted in a summary on the trends and methods in terrestrial isopod ecology (Hornung et al. 1992). The last meeting MRW participated in was in 1997, in Haifa Israel. This symposium was jointly organized by MRW himself and E. Hornung. The latest meeting attracted the isopodologists' crowd to Poitiers last summer (26–30. June, 2014). This meeting was a worthy tribute to Professor Michael Warburg's memory.

Scientific career and personal life of M.R. Warburg

Michael R. Warburg was born in Berlin, Germany in 1931. His family left the country in 1934 due to the Nazi threat. They moved first to London and, shortly after, to Haifa, then under British mandate, in Palestine. His reminiscences start in that period, from when he was five years old. MRW himself divided his memoir into periods which I have followed in this tribute (see also Hornung and Warburg 2014).

Childhood (1936–1946)

The family recalls that "*he was always collecting animals, and had all sorts of self-made cages for reptiles and other animals, which he kept on the roof of the house. Though Haifa was a city, in those days you didn't need to go far in order to reach the natural environment, it was right there, and he used to spend most of his time outside*". He himself mentions in his memoir that "*Already as a young boy, I used to walk with my late father, an ardent naturalist at heart, though to his regret not by profession, in the fields and woods on Mt. Carmel where we lived*". He often mentioned his great walks while bird watching with his father on the marshy plains (now housing estates) around Mt. Carmel. He dedicated his book on isopods (Warburg 1993) to his father and uncle: "*In memory of my late father,* Sigmund*, and uncle,* Edgar*, who both influenced in different ways my approach to nature*".

Graduate studies at the Hebrew University (1950–1954)

His university studies started with some difficulties, as he failed the preliminary biology exams and had to start his first semester in the Faculty of Mathematics. Still, from time to time he was able to attend biology lectures. During the second semester he was allowed to choose a few laboratory practicals and finally, at the end of the second year he transferred to major in biology. In his third year he received an assistantship on the entomology course. He started to work on his MSc thesis under Dr A. Zuckerman

on the life cycle of *Trypanosoma lewisii*, a blood parasite of rodents. His attempts to locate the parasites were not successful, so he moved to a different research project: producing a serum against *Plasmodium berghei*, a blood sporozoan lethal to hamsters. With time-consuming, diligent work ("*half a year's work which I did working days and nights*") he successfully finished the project in 1954, receiving the grade 'very good'. In 1955, he reached two milestones in his life: he published his first paper, and married Hava, his wife for the next 59 years.

Teachers Seminary Oranim (1955–1956)

He was appointed to a position in Oranim, in the Teachers' Seminary. "*Throughout the entire period I have conducted field trips in the country and have collected isopods. My main difficulty was how to identify them*." He made every effort to familiarize himself with isopod identification using Vandel's (1960, 1962) papers on isopod taxonomy. He also sought help from two scientists at the Vienna Naturhistorisches Museum, Dr. F. Strouhal, and Dr. K. Schmölzer, experts on the western Mediterranean isopod fauna. MRW himself never became a taxonomist, instead, he was more interested in the physiology, morphology and ecology of isopods.

While teaching in Oranim, he came across Edney's (1954) review paper on woodlice and their land habitat. He decided to find an university where he could conduct research in this topic for his Ph.D. thesis. In 1956 he was accepted into the graduate program at Yale University, in the USA.

Graduate studies at Yale University, New Haven, Connecticut, US (1956–1960)

In his application he outlined three research plans and "*Yale University offered both a fellowship and the choice of one of the three subjects for research towards a Ph.D. suggested by me…*". He decided on the 'The ecological, behavioral and physiological adaptations of terrestrial isopods' with Prof. G.E. Hutchinson as his supervisor. First, he started with physiological and behavioral studies in the lab on local species, then switched to work on a desert species, *Venezillo arizonicus*. The research focused on adaptations to different microhabitats and microclimatic conditions such as relative humidity and temperature, using thermo-hygrograms. He quantified the behavioral responses of isopods to such conditions using a choice chamber / thermo-preference apparatus. He concluded that the interaction of three environmental factors, temperature, humidity and light, explain the majority of microhabitat choices of terrestrial isopods. He published his results on physiological ecology, specifically on water balance (evaporative water loss) and thermal balance of isopods in four papers (nos: 1-4).

After the completion of his Ph.D. in 1960, he returned to Israel and began looking for a job. It was not easy but finally he got an one-year position at the Tel Aviv University.

Tel-Aviv University (1960–1961)

MRW had limited facilities there, but he was able to conduct extensive field work, collecting isopods in all parts of the country. Field work was an essential part MRW's professional life. He summarized 80 years of isopod collecting efforts, 40 years of which was his own, in a review paper in 2007 (no. 45). Collecting sites in over 600 localities were visited approximately 900 times, resulting in a total of 41 species records for Israel. The collection is now owned by the university in Tel Aviv.

University of South Australia (1962–1964)

In 1962 MRW was awarded a Senior Research Fellowship at the Zoology Department, University of Adelaide, South Australia. Although his research there, under the supervision of Prof. H.G. Andrewartha, focused on the ecology of *Tiliqua rugosa*, a large skink, he managed to study woodlice as well. He conducted experimental work on evaporative water loss and thermal balance of isopods under controlled conditions. This fellowship resulted in eight papers, two of them on terrestrial isopods. (Nos 5-6).

He returned back to Israel in 1964 and, after several temporary jobs, he received a position at the Israel Institute for Biological Research (Ness Ziona, near Tel Aviv).

Israel Institute for Biological Research (1965–1972)

Here, he studied the cave tick, *Ornithodorus tholozani*, specifically its neurosecretory cells, reproduction and ecology. He published 11 papers on this topic. He learned techniques in neurosecretion at Sheffield University, under Prof. K. Highnam and in Paris, under Prof. M. Gabe, and utilized this knowledge in isopod research (No 10). He continued collecting isopods extensively. This time he had professional help from Dr. H. Schmalfuss (Natural History Museum, Stuttgart, Germany), who both verified MRW's identifications and identified several new species, naming one of them after MRW: *Chaetophiloscia warburgi* Schmalfuss, 1991.

TECHNION (1972–2013)

He spent his last 40 years at the Israeli Institute for Technology (TECHNION), Haifa. His papers published during this period can be grouped mainly into ecological, ecophysiological and behavioral topics (Fig. 1). These themes overlap in his publications as he used diverse methods and approaches to describe the phenomena in question.

One research focus was species diversity and distribution mainly in the northern part of the Mediterranean region, where several different localities differ in plant and stone coverage and in climatic conditions. MRW was also interested in the intra-habitat

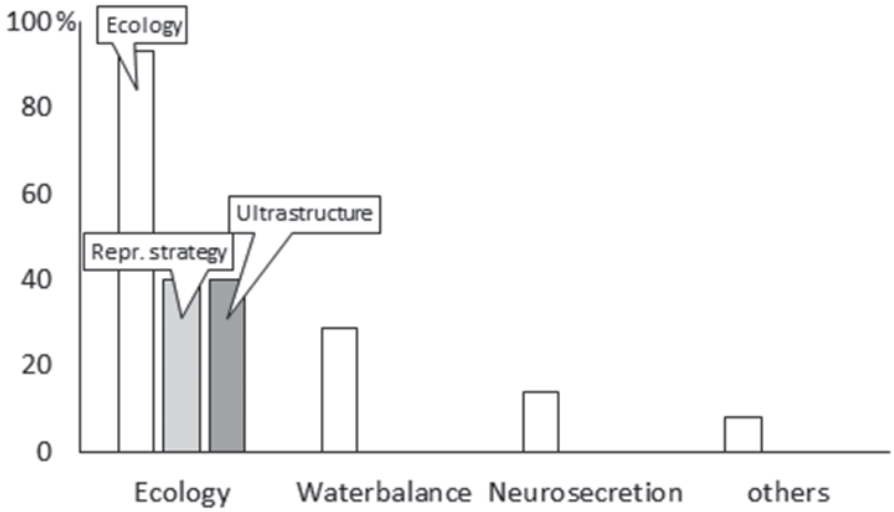

Figure 1. The distribution of MR Warburg's papers on isopods by topic.

dispersion of woodlice, specifically how the number and distribution of potential sheltering sites influenced the presence of isopods in different types of habitats.

The second field of MRW's interest was population and life history. He conducted research on the population structure, sex rate, life history and reproductive strategies of isopods. The latter included detailed studies of the structure of the female reproductive system and structure of the brood-pouch.

Behavioral studies were focused on responses of woodlice to temperature, light and humidity and resulted in two papers (nos: 1, 7).

In one of MRW's papers (no 2) he reported on osmolarity of isopods under different environmental conditions.

Later, during his sabbaticals he revisited his favourite places in the US and Australia and, in collaboration with colleagues such as Prof. M.A. Alikhan in Ontario, Canada; Prof. C. Crawford in New Mexico, US, and Prof. P. Greenaway, New South Wales, Australia, conducted new research on isopod ecology. In Haifa he kindly hosted several isopodologists, including the author and collaborated internationally in laboratory and field projects.

M.R. Warburg's scientific achievements

During his scientific career M.R. Warburg studied a broad range of animal taxa such as ticks, scorpions, amphibians, and reptiles in addition to terrestrial isopods (Fig 2). All these diverse projects fit within the disciplines of species diversity, distribution, ecophysiology, reproductive systems and strategies (Fig. 3). He published more than 180 papers, over 75 abstracts, 2 books and was the co-editor of the 4th Symposium on the Biology of Terrestrial Isopods volume.

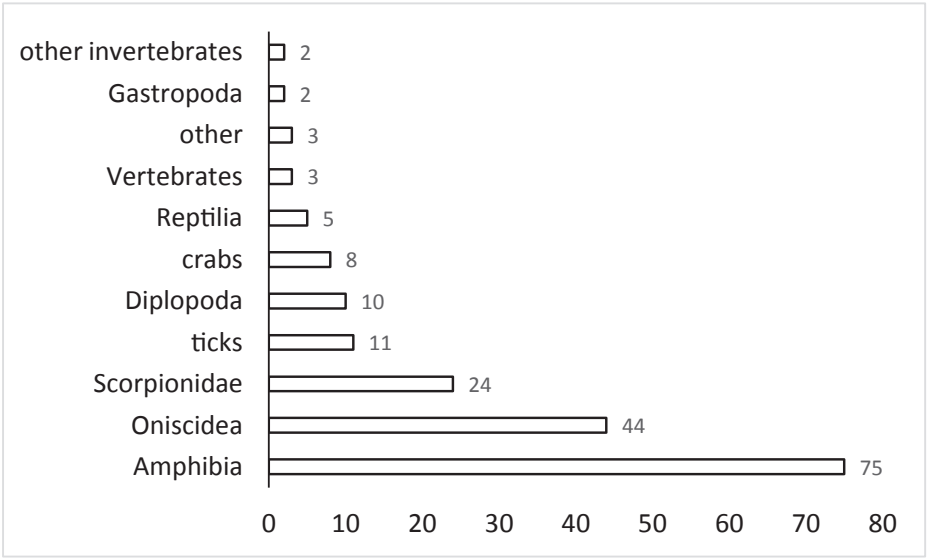

Figure 2. Distribution of published papers by animal groups.

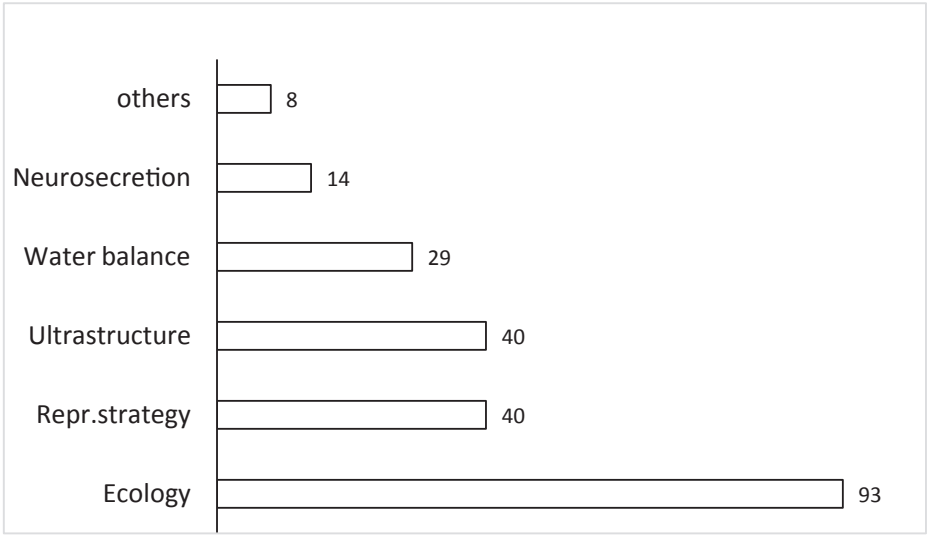

Figure 3. Percentage of MRW's publications falling into different topics. (One paper may cover several subjects.)

Even after his retirement MRW continued to be scientifically active. As Professor Emeritus, he focused on summarizing his results and sharing them with the scientific community. In the past 13 years he kept publishing; fourteen of these papers are (partial) reviews on his favorite taxa.

He is survived by his wife, Hava (a biology teacher) his son Ittai, his daughters Meirav and Sharon and 11 grandchildren (photo 13).

Acknowledgement

A major source for this tribute was MRW's own well organized memoir and publication list. My thanks go to the family, especially Hava and Sharon who supplied me with additional information about his life, sent photos of him and who encouraged me in the process of writing (Hornung and Warburg 2014).

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Budapest, 29th of April, 2015.

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1 In the company of his brother (Gaby) and sister (Hanne), 1932 **2** 1936 (5 years old) when his memoir started **3** With his father and sister on the field **4** Cages everywhere... With a pelican (1952) **5** Physiology lab (1954) **6** Israel Institute for Biological Research (1965) **7** With Hava Warburg in 1956 (married in 1955) **8** On a zoology excursion, 1954 **9** Santa Rita experimental station, USA, 1957 **10** 1990: Searching for Isopods. Sabbatical in New South Wales **11** 1997, Haifa Symposium, Farewell party **12** 1982: In New Zealand with Hava during sabbatical **13** The last family photo in 2010

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Patterns of taxonomic diversity among terrestrial isopods

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Abstract

The publication of the world catalog of terrestrial isopods some ten years ago by Schmalfuss has facilitated research on isopod diversity patterns at a global scale. Furthermore, even though we still lack a comprehensive and robust phylogeny of Oniscidea, we do have some useful approaches to phylogenetic relationships among major clades which can offer additional insights into isopod evolutionary dynamics. Taxonomic diversity is one of many approaches to biodiversity and, despite its sensitiveness to biases in taxonomic practice, has proved useful in exploring diversification dynamics of various taxa. In the present work, we attempt an analysis of taxonomic diversity patterns among Oniscidea based on an updated world list of species containing 3,710 species belonging to 527 genera and 37 families (data till April 2014). The analysis explores species diversity at the genus and family level, as well as the relationships between species per genera, species per families, and genera per families. In addition, we consider the structure of isopod taxonomic system under the fractal perspective that has been proposed as a measure of a taxon's diversification. Finally, we check whether there is any phylogenetic signal behind taxonomic diversity patterns. The results can be useful in a more detailed elaboration of Oniscidea systematics.

Keywords

Bodiversity, diversification, systematics, fractals, phylogeny, species richness, taxonomic asymmetry

Introduction

Terrestrial isopods constitute one of the most remarkable lineages of invertebrates that managed to conquer land. Modern species represent almost all evolutionary steps that enabled them to leave the marine environment and occupy almost the whole range of terrestrial habitat types. This makes them a unique case within global biodiversity and offers lots of opportunities to biological research, especially in fields like evolution, ecology and ecophysiology (Warburg 1993). Even though the phylogeny of terrestrial isopods has not yet been adequately resolved, the monophyly of certain large clades is well supported, and we do have a relatively good picture of the major evolutionary transitions that made possible the conquest of more and more arid habitat types.

Terrestrial isopods, the suborder Oniscidea within the order Isopoda, are currently considered to be a monophyletic taxon even though their monophyletic origin has been questioned in the past (see Schmidt 2008 for a review). The status of the family Tylidae might still be considered somewhat ambiguous but, in general, most authors agree that isopods invaded land from marine ancestors, most probably once in their history. They have evolved a number of unique adaptations with no parallels in other related taxa (see Hornung 2011 for a review), such as the water conducting system, the various forms of pleopodal lungs and the cotyledons in the marsupium. In fact, the last two structures might be considered as analogous to the lungs in vertebrates and the placenta in mammals, respectively.

From an ecological point of view, Oniscidea live in almost all biomes, having successfully invaded most areas of the world, with the exception of the poles and very high elevations (>4,800 m, Beron 1997). In some ecosystems they constitute one of the most important components of decomposer communities, being largely phytosaprophagous and often occurring at very high population densities (Dias and Hassall 2005). Some species that live in very harsh desert environments have even attained the level of subsocial organization. In addition, terrestrial isopods are amongst the most common and species-rich components of cave-dwelling animal groups with very large percentages of troglobitic species. Oniscidea also include amphibious and even aquatic species that have secondarily returned to live in salt lakes or subterranean freshwaters (Tabacaru 1999, Taiti and Xue 2012).

Oniscidea probably originated in the Carboniferous (Broly et al. 2013), and are represented today by more than 3,700 species belonging to more than 500 genera in 37 families and five higher clades (infraorders/sections) (Schmidt 2008). The long evolutionary history of terrestrial isopods has led to considerably asymmetric species-richness patterns among major clades. The more basal Diplocheta and Tylida are relatively species-poor, Mesoniscidae are represented by just two species, while the vast majority of species belong to the, more apical, sister clades Synocheta and Crinocheta, with the latter being more 'terrestrial' and by far the richest in species number. Such asymmetries in taxonomic richness among clades might reflect differences in evolutionary dynamics, such as the relative strength of evolvability and genetic constraints, or the roles of key innovations in the rates of lineage diversification. Therefore, the identifi-

cation of significant patterns in species richness within clades can provide important insights into the history of biodiversity.

The study of taxonomic diversity aims to explore such patterns across different taxonomic levels. Despite its sensitiveness to biases in taxonomic practice it has proved useful in exploring diversification dynamics in characteristic biota (e.g., Simberloff 1970), plant (Väre et al. 2003) or animal groups (Larson et al. 2001, Sierwald and Bond 2007, Pincheira-Donoso et al. 2013). Obviously, exploration of taxonomic diversity should be based on a comprehensive account of the species known within the respective lineage. The publication of the world catalog of terrestrial isopods some ten years ago (Schmalfuss 2003, 2004) has facilitated this line of research on isopod diversity patterns at a global scale, even though the isopod fauna of large parts of the world remains largely unexplored or, at least, inadequately known. Nevertheless, our current knowledge on the group can provide a relatively solid basis for a preliminary analysis of its taxonomic diversity that, in turn, might identify important gaps and other issues that should come into the focus of future research. Such an analysis can also contribute to the broader discussion on patterns of diversification rates, such as the possible tendency for exceptionally rich clades to be rare or the fractal structure of taxonomic levels (Burlando 1990).

In the present work we attempt an analysis of taxonomic diversity patterns among Oniscidea based on an updated world list of species, exploring species diversity at the genus and family level, as well as the relationships between species per genera, species per families, and genera per families. Even though the assignment of genus and family status for a group of species or clades is arbitrary, experts in each higher taxon usually follow a similar approach, so that the study of such patterns is still meaningful to a considerable extent. Also, we do know that several families (or even genera) of Oniscidea might not be monophyletic, and this could lead to uncertainties in results from such a taxonomic diversity analysis. Nevertheless, this kind of analysis actually helps towards identifying such problems. For example, the exploration of a possible fractal structure in the isopod taxonomic system, which has been proposed as a measure of diversification, is a useful tool for this, and we do address the issue herein. Finally, we check whether there is any phylogenetic signal behind taxonomic diversity patterns.

Methods

In order to compile a complete species list of valid isopod species, we used as a basis the world catalog published by Schmalfuss (2003) and its updated electronic version (Schmalfuss 2004). To this we added all taxonomic changes made till April 2014 (new species descriptions plus nomenclatural changes). For nomenclature and familiar assignments, we followed Schmidt (2008). Some problematic cases were identified as such and left outside further treatment. The final complete species list per family and genus was compiled after a thorough evaluation of each species' known status by the second author (ST). The complete list is available by the authors upon request.

Species description rates were calculated from dates appearing in current nomenclature.

For phylogenetic information, we used the morphological analysis by Erhard (1998) and Schmidt (2008), and the molecular analysis by Mattern and Schlegel (2001).

In order to test for skewness in the frequency distribution of species richness at different taxonomic levels we used the standardized skewness metric and the Shapiro Wilk test for deviation from normality. In all other analyses we applied standard linear regressions and the Pearson product moment, or the Spearman rank correlation coefficient.

Results

In the ten years after the publication of the electronic version of the world catalog of terrestrial isopod species (Schmalfuss 2004), less than 80 new species were added. In particular, 3,637 species were included in the 2004 list, while 3,710 species are recognized in our 2014 (as of April) compilation. These species belong to 527 genera and 37 families. The complete list includes 192 species of ambiguous generic assignment and 37 genera (that include 90 species overall) of ambiguous familial assignment.

The list of families with the respective numbers of genera and species is given in Table 1.

The family with the highest species richness is Armadillidae, followed by Philosciidae and Trichoniscidae. The same three families are also the richest in genera, albeit in a different order, with Philosciidae first, followed by Trichoniscidae and Armadillidae. There are seven monogeneric families, four of them with monotypic genera. Species richness is significantly correlated with genera richness (Spearman rank correlation coefficient: $r_s = 0.91$, $p < 0.001$).

Species descriptions per decade showed a bimodal distribution in the last century with most of the currently valid species being described either in the first half of the 20th century, especially in the '20s, or from 1960 to 1990 (Fig. 1A). The cumulative number of species seems to have reached a plateau in the last two decades (Fig. 1A).

Frequency distributions of isopod richness are significantly right-skewed (for genera within families: skewness = 6.82, Shapiro-Wilk test $p < 0.001$; for species within genera: skewness = 62.3, Shapiro-Wilk test $p < 0.001$; for species within families: skewness = 5.7, Shapiro-Wilk test $p < 0.001$). This means that most families and genera consist of few genera and species, respectively, while very rich lineages are rare (Fig. 2).

The number of species per genus in a family is not predicted by the number of genera per family (Fig. 3A). On the other hand, the number of species per family is positively correlated with the number of genera per family ($r = 0.90$, $p < 0.001$; Fig. 3B).

The frequency of genera is negatively correlated with the number of species per genus (in logarithmic space: $r = -0.88$, $p < 0.001$; Fig. 4), giving a fractal dimension (= the absolute value of the slope of the respective linear regression) of 1.02, which becomes 1.14 when unit values are excluded from the analysis (to avoid the long tail of zeros, i.e., very large genera).

Table 1. List of families with their respective numbers of genera and species, the latter separately for those in known genera and those of uncertain generic assignment.

Family	Number of genera	Species in known genera	Species of uncertain generic assignment	Total
Armadillidae	80	579	118	**697**
Philosciidae	107	501	36	**537**
Trichoniscidae	87	492	2	**494**
Porcellionidae	19	326	7	**333**
Armadillidiidae	14	256	0	**256**
Eubelidae	50	253	2	**255**
Agnaridae	14	157	10	**167**
Platyarthridae	7	122	0	**122**
Trachelipodidae	6	109	4	**113**
Scleropactidae	26	107	0	**107**
Ligiidae	6	95	0	**95**
Styloniscidae	10	81	1	**82**
Cylisticidae	5	66	0	**66**
Detonidae	4	39	0	**39**
Halophilosciidae	3	35	0	**35**
Oniscidae	5	31	10	**41**
Alloniscidae	2	23	2	**25**
Tylidae	2	22	0	**22**
Spelaeoniscidae	7	20	0	**20**
Delatorreidae	3	18	0	**18**
Dubioniscidae	3	15	0	**15**
Rhyscotidae	2	13	0	**13**
Olibrinidae	4	11	0	**11**
Scyphacidae	2	11	0	**11**
Bathytropidae	1	10	0	**10**
Balloniscidae	2	8	0	**8**
Titaniidae	5	6	0	**6**
Tendosphaeridae	3	4	0	**4**
Stenoniscidae	2	4	0	**4**
Pudeoniscidae	2	4	0	**4**
Irmaosidae	1	2	0	**2**
Mesoniscidae	1	2	0	**2**
Schoebliidae	1	2	0	**2**
Berytoniscidae	1	1	0	**1**
Bisilvestriidae	1	1	0	**1**
Hekelidae	1	1	0	**1**
Turanoniscidae	1	1	0	**1**
Unknown	37	90	0	**90**
Total	527	3,518	192	**3,710**

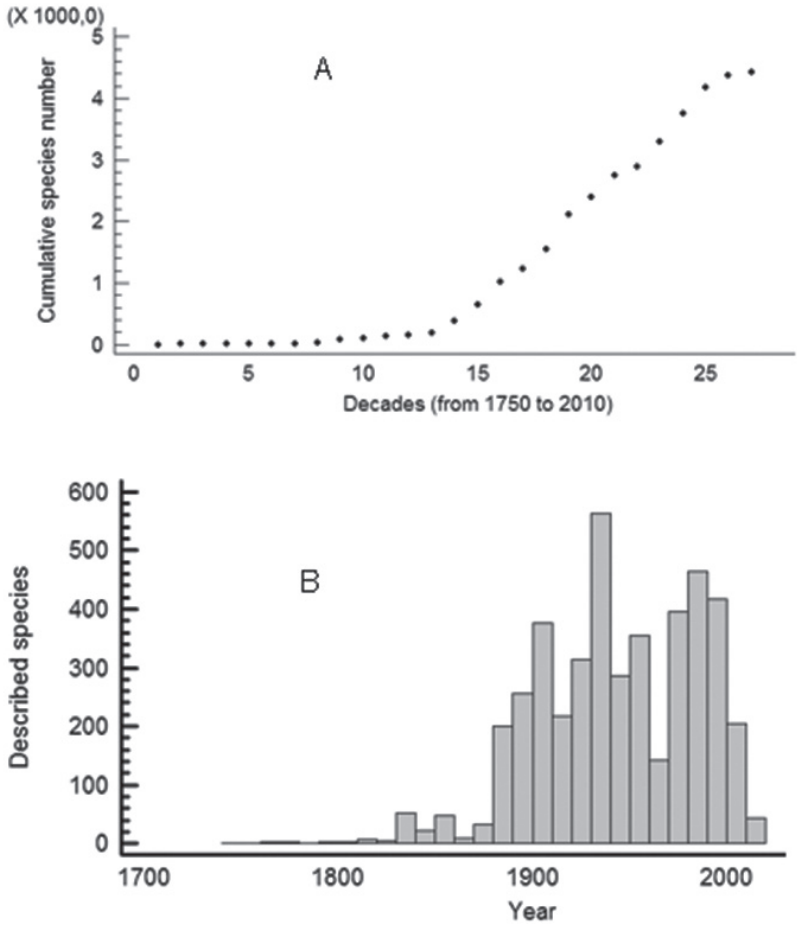

Figure 1. Rate of isopod species description since 1750. **A** Number of terrestrial isopod species described per decade **B** Cumulative species number of terrestrial isopods per decade, since 1750.

Species richness values per family were mapped on the available phylogenetic trees for Oniscidea (Fig. 5A, B) to see whether there is any apparent phylogenetic signal in richness patterns. It is obvious that basal clades are poor, but inside the more derived clade Crinocheta-Synocheta the picture is not very clear (Fig. 5A). Nevertheless, inside Crinocheta (Fig. 5B) it seems that species richness is much higher in the more derived clades.

Discussion

Terrestrial isopods are the largest suborder of Isopoda and actually the only group of Crustacea that has managed to exploit almost the whole range of terrestrial ecosystems. The ca. 3,700 species known so far include clades that have evolved a variety of mor-

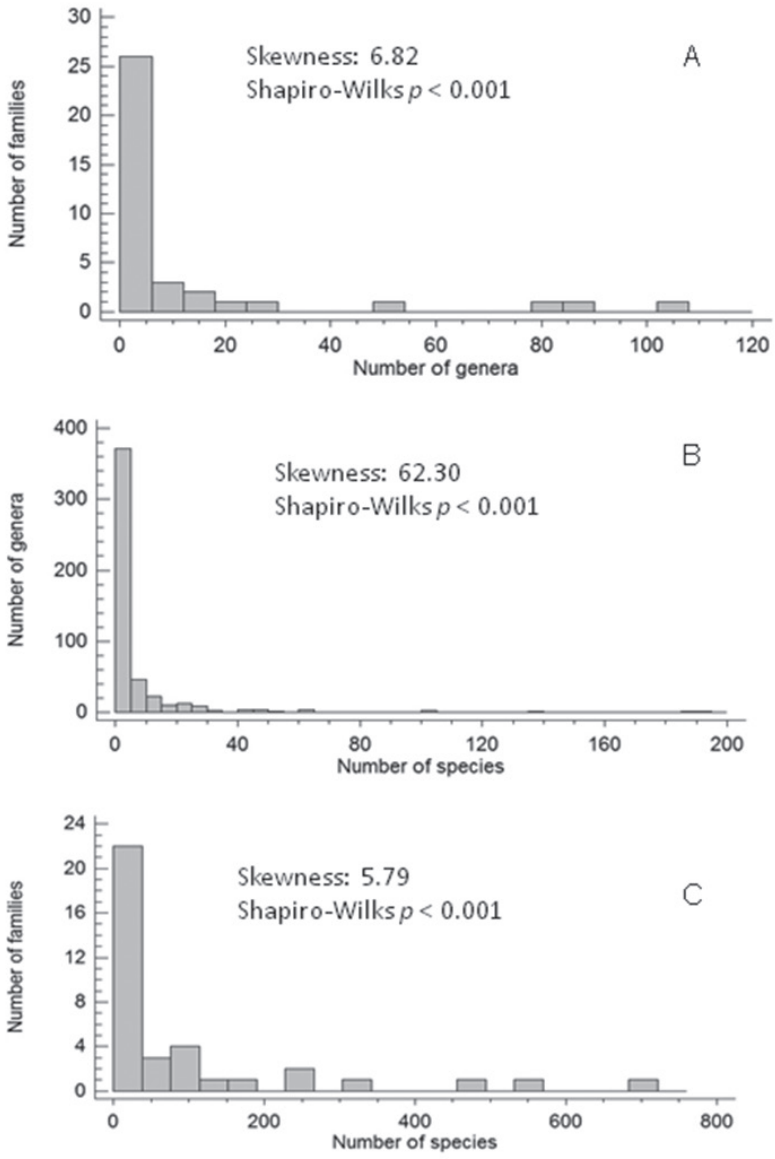

Figure 2. Skewness in the distribution of taxonomic richness with results of the respective Shapiro-Wilks tests. **A** for number of genera per families **B** for number of species per genera, and **C** for number of species per families.

phological, physiological and behavioral characters offering unique solutions to key problems pertaining to the adaptation to the life on land, so that today they represent almost all transitional stages from marine to extremely arid environments.

According to the rate of species descriptions presented herein one might assume that the vast bulk of the global oniscid diversity has been known, and the total rich-

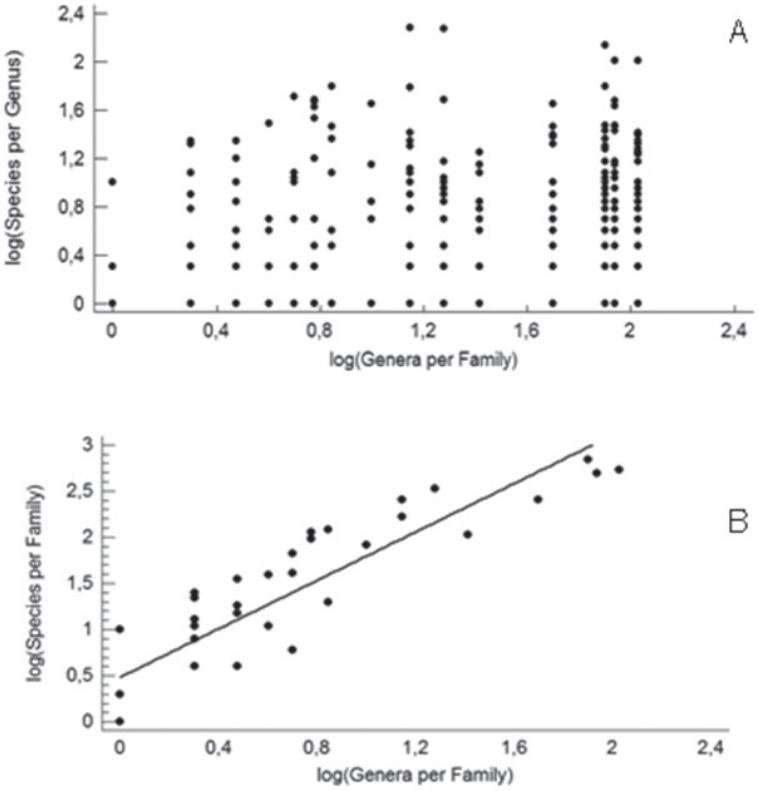

Figure 3. Regression of species richness per higher taxonomic groups against richness of genera per families (all in logarithmic values). **A** species per genera **B** species per family.

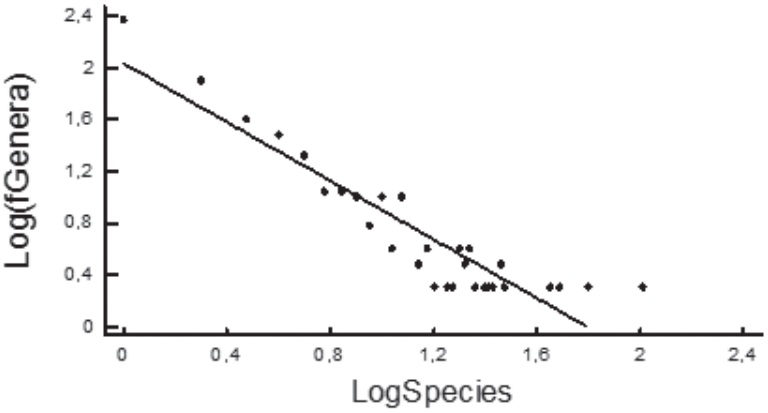

Figure 4. Linear regression of the frequency of genera (fGenera) against their respective species richness, revealing the fractal nature of terrestrial isopod taxonomy. The slope of the regression (1.14) gives the fractal dimension. Unit frequency values – genera with unique values of species richness – have been excluded in order to avoid a long queue of zeros that smoothens the slope only due to the fact that the size of large genera is more probable to be unique.

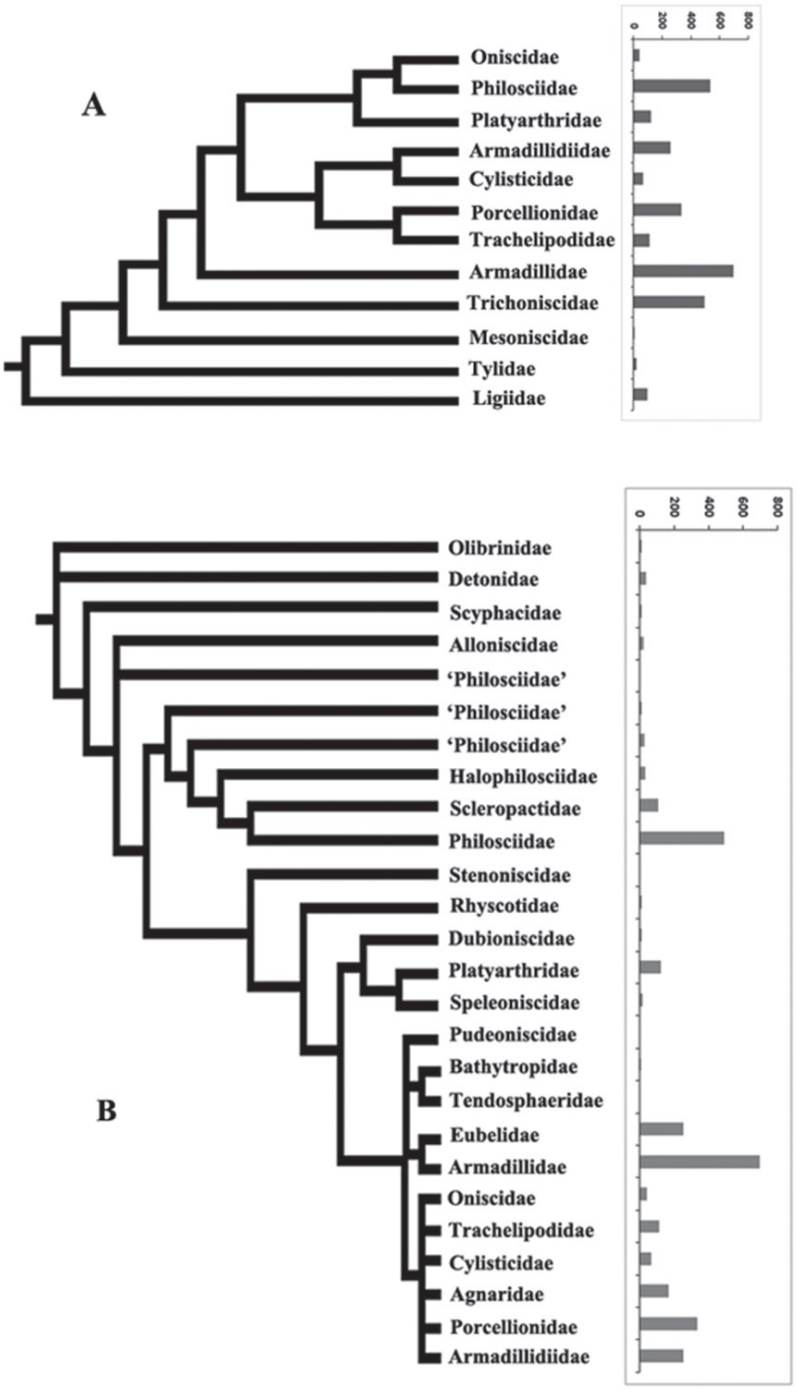

Figure 5. The species richness of Oniscidea families against two phylogenetic hypotheses. **A** Tree from Mattern and Schlegel (2001) incorporating also hypotheses from Erhard (1998) **B** tree for Crinocheta from Schmidt (2008).

ness will not change to a significant degree in the near future. Nevertheless, we should note that the 'plateau' in the accumulated species richness observed in the last two decades might be better attributed to the decline in taxonomic expertise on the group. Indeed, there are very few active taxonomists of Oniscidea today. A large part of the world remains unexplored, especially the tropics, and the current trends in funding and 'academic prestige' do not leave much space for optimism that they will be explored soon. It is equally important to note that many thousands of caves around the world are expected to host hundreds, if not thousands, of isopod species, taking into account that Oniscidea are amongst the richest animal taxa in troglobitic species, most of which occur in one or a few local caves and/or other subterranean habitats. Furthermore, several analyses based on molecular markers reveal an even higher diversity among isopod taxa (e.g., Klossa-Kilia et al. 2006, Parmakelis et al. 2008, Hurtado et al. 2010, 2013, Kamilari et al. 2014). With the increasing application of molecular techniques in isopod phylogeny and taxonomy, we expect that more and more 'cryptic' species will be found. A reasonable estimation of the expected global richness of Oniscidea might be between 5,000 and 7,000 species. From a biogeographical perspective, and despite the reasonable bias in Europe due to the geography of research and researchers, it still remains true that the Mediterranean region is particularly rich in Oniscidea and the same is true also for other areas of the world with Mediterranean-type ecosystems, such as South Africa and western Australia. Furthermore, terrestrial isopods are highly diversified also in tropical regions, especially in areas with increased environmental heterogeneity. We still lack a concise biogeographical analysis of Oniscidea at a global scale, but all evidence from narrower geographical scales show that isopod richness is highly correlated with geographical and landscape complexity (e.g., presence of islands, cave systems, mountainous regions) and environmental heterogeneity (e.g., Mediterranean).

The analysis of global diversity conducted herein reveals a strong right-skewed frequency distribution, so that Oniscidea mostly contain genera with few species and families with few genera. This is a pattern observed also in other animal taxa (e.g., hexapoda: Mayhew 2001, mammals: Purvis et al. 2011, reptiles: Pincheira-Donoso et al. 2013) suggesting that even though very diverse lineages are rare, they contribute significantly to the total diversity of each higher taxon. In fact, patterns of taxonomic diversity identified for Oniscidea show more similarities with those in other organisms, suggesting that taxonomic structure might not be idiosyncratic for each higher taxon. This might be related to the critical role of key innovations in clade diversification, such as improved resistence towards dehydration that enabled Crinocheta to conquer new 'macro-niches', such as habitats with less relative humidity, more arid habitats etc. (see Schmalfuss 1998).

The correlation between number of species and number of genera in a family, in combination with the fact that numbers of species per genus cannot predict generic richness in a family, underlines the wide variation inside Oniscidea. This is because in addition to the somewhat trivial fact that many small genera – even monotypic – may be found in large families, the pattern is also based on the occurrence of very diverse genera in small families.

If fractal geometry of taxonomic systems indeed reflects real patterns of evolutionary diversification, then isopod diversity appears underestimated. The 'fractal dimension' of most arthropods and North American isopods (including freshwater and marine) is 1.50 according to Burlando (1990), while for Aegean terrestrial isopods it is 1.48 (Sfenthourakis 1994). Lower values suggest either lower diversification or the need for further splitting of large taxa. Given that diversification cannot be considered low in Oniscidea, the 1.14 value found herein implies that large genera might not be monophyletic units and need to be split into smaller ones. The possible non-monophyly of many higher taxa inside Oniscidea is a suggestion regularly reported in the relevant literature, and recent molecular analyses seem to also support such statements (e.g., *Hemilepistus*: Dimitriou, Kashani and Sfenthourakis, in preparation).

An intriguing question refers to the role of 'key innovations' in adaptive radiations, which would lead to prominent radiations in clades that have acquired some new feature offering significant selective advantages. Such a pattern would lead to very asymmetric phylogenies and key innovations could be mapped as defining synapomorphies of prolific clades. If the phylogeny of Schmidt (2008) proves to be correct, then such a case should be exemplified by the derived clade including the 'highly terrestrial' families Eubelidae, Armadillidae, Armadillidiidae, Porcellionidae, Trachelipodidae, Agnaridae, Cylisticidae and Oniscidae. On the other hand, the high diversity of Trichoniscidae and Philosciidae does not seem to conform to this pattern, unless some not currently obvious unique key innovation can be identified in these taxa in the future. An alternative explanation is that these families are not monophyletic, so the total richness of the actual monophyletic units produced from their split would be lower, but even then their richness would still be high enough to ask for an explanation.

It is absolutely necessary to have a robust phylogeny of Oniscidea families (and genera) in order to gain crucial insights into the evolution of this fascinating taxon. New techniques using Next Generation Sequencing can facilitate this task and provide very useful information.

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RESEARCH ARTICLE

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The terrestrial Isopoda (Crustacea, Oniscidea) of Rapa Nui (Easter Island), with descriptions of two new species

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Abstract

Nine species of terrestrial isopods are reported for the Polynesian island of Rapa Nui (Easter Island) based upon museum materials and recent collections from field sampling. Most of these animals are non-native species, but two are new to science: *Styloniscus manuvaka* **sp. n.** and *Hawaiioscia rapui* **sp. n.** Of these, the former is believed to be a Polynesian endemic as it has been recorded from Rapa Iti, Austral Islands, while the latter is identified as a Rapa Nui island endemic. Both of these new species are considered 'disturbance relicts' and appear restricted to the cave environment on Rapa Nui. A short key to all the oniscidean species presently recorded from Rapa Nui is provided. We also offered conservation and management recommendations for the two new isopod species.

Keywords

Crustacea, Isopoda, Oniscidea, new species, Rapa Nui, Easter Island, disturbance relicts, caves

Introduction

Rapa Nui (Easter Island) is one of the most ecologically degraded islands in Polynesia. A number of factors including geographic isolation, island size and low topographic relief (Rolett and Diamond 2004) predisposed Rapa Nui to dramatic human-induced environmental change. Between Polynesian colonization (800–1200 CE; Hunt and Lipo 2006, Shepardson et al. 2008) and prior to European contact in 1722 (McCall 1990), a catastrophic ecological shift occurred where the palm-dominated shrubland shifted to grassland (Wynne et al. 2014). By the mid-nineteenth century, most of the island was converted into a century-long sheep-grazing operation (Fischer 2005).

Contemporarily, few native plant species remain and all terrestrial vertebrates have gone extinct (Wynne et al. 2014). Researchers have described the arthropod communities of Rapa Nui as being equally impoverished (Kuschel 1963, Campos and Peña 1973, Desender and Baert 1997). Of the nearly 400 known arthropod species, only 30 species (~5%) have been identified as either endemic or indigenous with the remaining species either intentionally or accidentally introduced to the island (Wynne et al. 2014, Bernard et al. 2015).

Through fieldwork led by the second author, at least eight island endemic and two Polynesian endemic arthropod species have been recently identified (Wynne et al. 2014). These include one psocopteran (Mockford and Wynne 2013), six species of collembolans (including five new species and one Polynesian endemic; Bernard et al. 2015), one recently described collembolan later identified as endemic (Jordana and Baquero 2008; Wynne et al. 2014), and the two new species of terrestrial isopods described in this paper. All of these animals are presumed to be cave-restricted and represent disturbance relicts – organisms now restricted to a fraction of their former range due to extensive anthropogenic disturbance (Wynne et al. 2014). Given that one-third of the island's endemic arthropod fauna appear restricted to the cave environment, this offers a unique opportunity for conservation and management.

With the exception of the Hawaiian islands (Taiti and Ferrara 1991, Taiti and Howarth 1996, 1997, Taiti 1999, Rivera et al. 2002, Taiti et al. 2003, Santamaria et al. 2013), terrestrial isopods from Polynesia are poorly known (see Jackson 1941 for a review). For Rapa Nui, only three species of terrestrial isopods were previously recorded (Fuentes 1914): *Ligia exotica* Roux, 1828, *Porcellio scaber* Latreille, 1804, and *Armadillidium vulgare* (Latreille, 1804). All of these isopods are non-native species. The purpose of this paper is to identify the terrestrial isopod fauna of Rapa Nui, including the descriptions of two new species.

Material and methods

Study area

Fieldwork was conducted on the Roiho lava flow, ~5 km north of the village of Hanga Roa during three research trips in 2008, 2009 and 2011. The study area is characterized

by gently rolling hills (i.e., extinct scoria cones) with coastal cliff faces flanking the western-most boundary. Vegetation was grassland and invasive guava (*Psidium guajava*) shrub. Within the collapse pit and skylight entrances of most caves, several non-native tree species occurred, including fig (*Ficus* sp.), avocado (*Persea americana*), apple banana (*Musa* × *paradisiaca*), roseapple (*Syzygium jambos*), guava (*Psidium guajava*) and *Eucalyptus* spp.

The cave environment

Caves are zonal environments often consisting of four principle zones: (1) an entrance (or light) zone representing a combination of both surface and cave climatic conditions; (2) a twilight zone where light is diminished and surface climate conditions are progressively dampened; (3) a transition zone characterized by complete darkness with a further diminished influence of surface climate conditions; and, (4) a deep zone (usually the deepest portion of the cave) where environmental conditions (e.g., complete darkness, temperature, and air flow) remain relatively stable over time and the evaporation rate is negligible (Howarth 1980, 1982). For each isopod detected within caves, we provide a zone designation in the "type material examined" section.

Sampling

Cave and surface sampling was conducted. Research teams (led by the second author) systematically sampled 10 caves during three research trips (16–21 August 2008; 28 June–17 July 2009; and 01–07 August 2011). Four methodologies (pitfall traps, time-constrained searches, opportunistic collecting, and timed direct intuitive searches) were applied to sample 10 caves during the first two trips. Pitfall trap construction consisted of two 946-ml stacked plastic containers (13.5 cm high, 10.8-cm-diameter rim and 8.9-cm base). A teaspoon of peanut butter placed in the bottom of the exterior container was used as bait. The bottom of the interior container had several dozen holes to allow the bait to "breathe" to attract arthropods. Traps were deployed for three to four days.

Time-constrained searches involved estimating a one-meter radius around each pitfall trap sampling station and then conducting a timed search. Searches were conducted for one to three minutes (one minute if no arthropods were observed, three if arthropods were detected) before pitfall trap deployment and prior to trap removal.

Opportunistic collection involved collecting arthropods as encountered – while deploying and removing pitfall traps, and between timed searches. During these intervals, personnel searched the ground, walls and ceilings as they walked the length of each cave. In five caves (where all the collecting methodologies were applied), we also conducted timed direct intuitive searches (DIS) of fern-moss gardens by gently combing through the fern and moss and looking beneath rocks for 40 search-minutes

per garden (two observers × 20 minutes per observer). In four additional caves, we limited sampling to DIS within fern-moss gardens only (two observers × 20 minutes per observer).

During the last research trip to the island, the deep zones of four of the caves were sampled via bait sampling and DIS. Three types of baits were placed directly on the ground and within cracks and fissures on cave walls, ceilings and floors: sweet potato (*Ipomoea batatas*), chicken and fish entrails, and small branches from local hibiscus (*Hibiscus rosa-sinensis*) and Gaoho (*Caesalpinia major*) shrubs. Two to three stations of each bait type were deployed, for four to five days, within the deep zone(s) of each cave. At proximity to bait sampling arrays, we also conducted one DIS by searching the cave floor for 10 minutes within a 1-m^2 area.

From 28 June through 08 July 2009 (total of 10 days), we deployed two 15 x 20 meter surface pitfall trapping grids. Surface Grid 1 (with trap numbers 1 - 20) was established inland at the approximate center of our study area. Surface Grid 2 (with trap numbers 21 - 40) was deployed at the western extent of the study area (~250 m from the coastal cliff face). All pitfall traps were countersunk to ground surface with trap spacing at 5 m between each trap.

For additional information on sampling refer to Wynne et al. (2014) at: http://www.bioscience.oxfordjournals.org/lookup/suppl/doi:10.1093/biosci/biu090/-/DC1

Cave codes

We recognize standard practice for locality information is to provide geographical coordinates to facilitate future collecting and interpretation. However, Chilean park officials have requested that neither cave names nor coordinates be included due to cultural and natural resource sensitivities of caves. In place of cave names, we used cave codes supplied by CONAF – Parque Nacional Rapa Nui. A copy of this paper, which includes a table of cave names with associated cave codes, is on file with CONAF – Parque Nacional Rapa Nui headquarters Hanga Roa, Easter Island, and CONAF, Jefe Departamento, Diversidad Biológica, Gerencia de Areas Protegidas y Medio Ambiente, Santiago, Chile.

Preservation, mounting, observation

All material was preserved in 95% ethanol. Identifications were based on morphological characters with the use of micropreparations. Line drawings were made with the aid of a camera lucida mounted on Wild M5 and M20 microscopes. Whole-specimen images were captured using a 1.1 MP Canon 5D Mark II (with a 65 mm zoom lens) mounted on a Visionary Digital BK Lab Plus camera mounting system. We used the program Zerene Stacker to merge images into a composite image. Photoshop CS5 was used for image post-processing.

Museum abbreviations

AMNH American Museum of Natural History, New York, USA;
BPBM Bernice P. Bishop Museum, Honolulu, Hawai'i, USA;
MNHN Museo Nacional de Historia Natural, Santiago, Chile;
MZUF Museo di Storia Naturale, sezione di Zoologia, dell'Università di Firenze, Florence, Italy;
YPM Peabody Museum of Natural History, Yale University, New Haven, Connecticut, USA.

Systematic account

Family Ligiidae
Genus *Ligia* Fabricius, 1798

***Ligia exotica* Roux, 1828**

Ligyda exotica; Fuentes 1914: 315.

Remarks. The record of this species by Fuentes (1914) needs to be confirmed since this littoral species has often been confused with other species in the past. Unfortunately, no specimens of *Ligia* have been recently collected from Rapa Nui, most likely due to lack of investigations along the littoral zones of the island.

Distribution. Pantropical.

Family Styloniscidae
Genus *Styloniscus* Dana, 1853

***Styloniscus manuvaka* sp. n.**
http://zoobank.org/00706DDB-9E9C-4DEF-AEA5-E9289287B7AB
Figs 1A, 2–4

Styloniscus sp.; Wynne et al. 2014: 713, 714, fig. 2b.

Type material examined. Chile, Rapa Nui: 1 ♂ holotype, 2 ♂♂, 2 ♀♀, 1 juv. paratypes (MNHN), Mahunga Hiva Hiva, Cave Q15-070, fern-moss garden (entrance zone), direct intuitive search, 10.VII.2009, leg. J.J. Wynne; 2 ♂♂, 1 ♀, 2 juvs. paratypes (MZUF), same location, 50 m from entrance, direct intuitive search (on decomposing tree branches; twilight zone), 6.VIII.2011, leg. J.J. Wynne; 1 ♀ paratype (MNHN), same data; 1 ♂, 1 ♀ paratypes (BPBM), Mahunga Hiva Hiva, Cave Q15-074, skylight entrance (1st entrance NE of main entrance; entrance zone), 3.VII.2009,

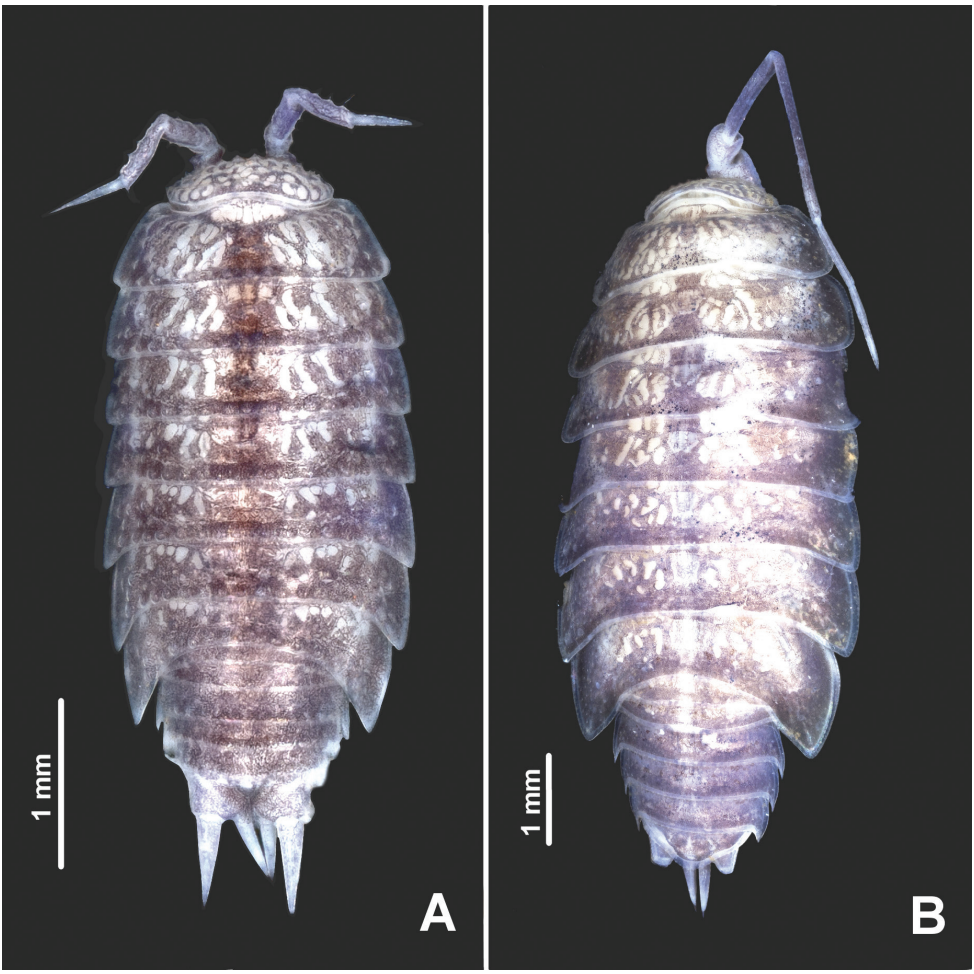

Figure 1. *Styloniscus manuvaka* sp. n.: **A** ♀ paratype in dorsal view. *Hawaiioscia rapui* sp. n.: **B** ♀ paratype in dorsal view.

leg. J.J. Wynne; 1 ♀ paratype (BPBM), Mahunga Hiva Hiva, Cave Q15-119, timed search at trap 4A, 5.VII.2009, leg. J.J. Wynne; 1 ♂ paratype (BPBM), same location, Zone 2 (approx. cave deep zone), trap, fish entrails 1, 6.VIII.2011, leg. J.J. Wynne; 1 ♀ paratype (BPBM), Mahunga Hiva Hiva, Cave Q15-071, Zone 2 (approx. cave deep zone), bait trap, fish entrails 1, 7.VIII.2011, leg. J.J. Wynne; 1 ♀ paratype (BPBM), Cave Q15-067, fern-moss garden (entrance zone), direct intuitive search, 4.XII.2008, leg J.J. Wynne.

Additional material examined. French Polynesia, Bass Islands (Austral Islands), Rapa Iti Island: 4 ♂♂ (YPM), Pumarua-Maurua Ridge, Pumarua and some west, 500-620 m, from dead leaves of the bird's nest fern, *Asplenium nidus*, 9.I.1980, leg. G. Paulay.

Description. Maximum length: ♂ 4 mm, ♀ 4.2 mm. Dorsum brown with the usual yellow muscle spots (Fig. 1A). Body ovoid with pleon narrower than pereon

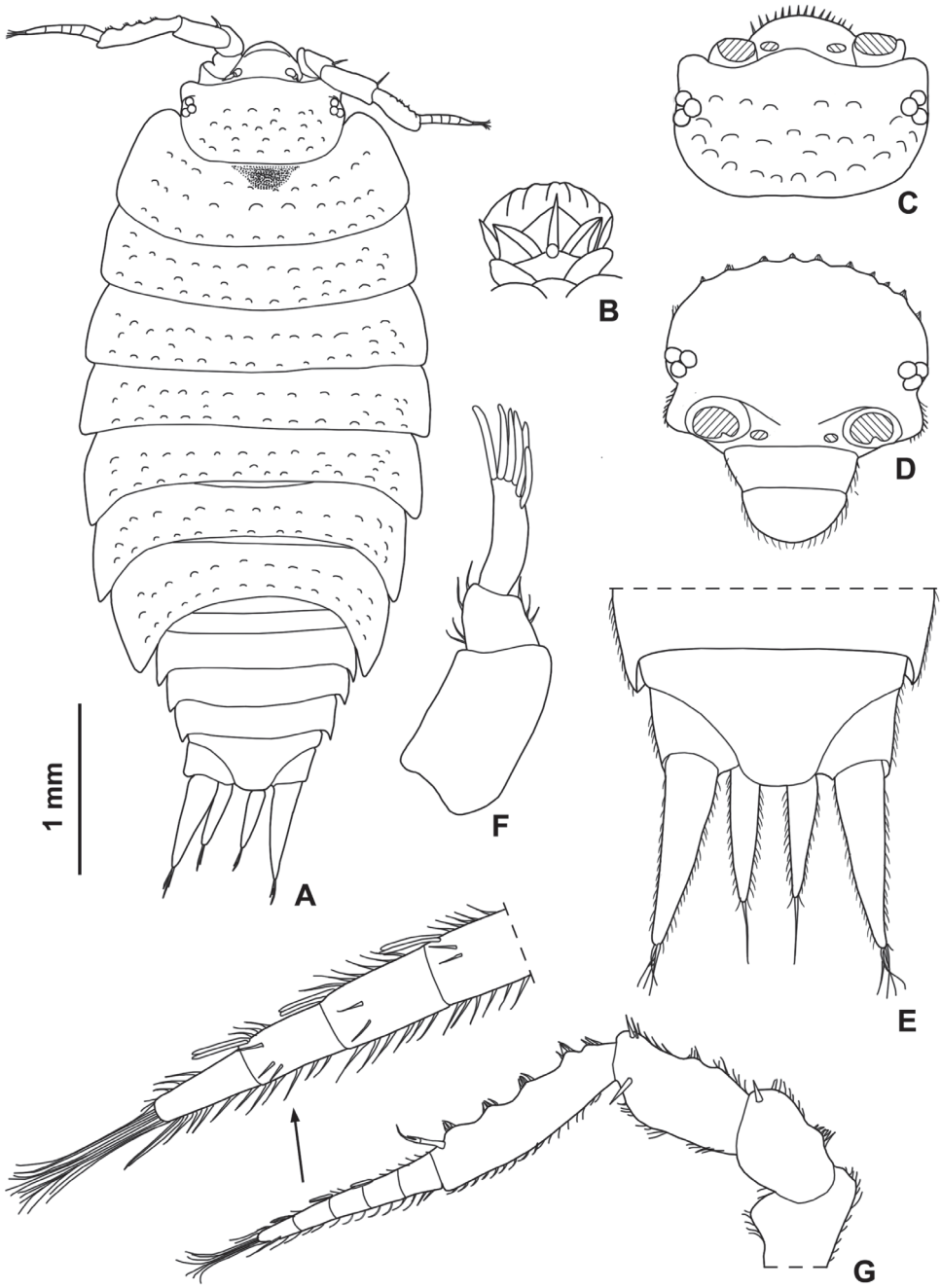

Figure 2. *Styloniscus manuvaka* sp. n., ♀ paratype: **A** adult specimen in dorsal view **B** dorsal scale-seta **C** cephalon in dorsal view **D** cephalon in frontal view **E** pleonite 5, telson and uropods **F** antennula **G** antenna.

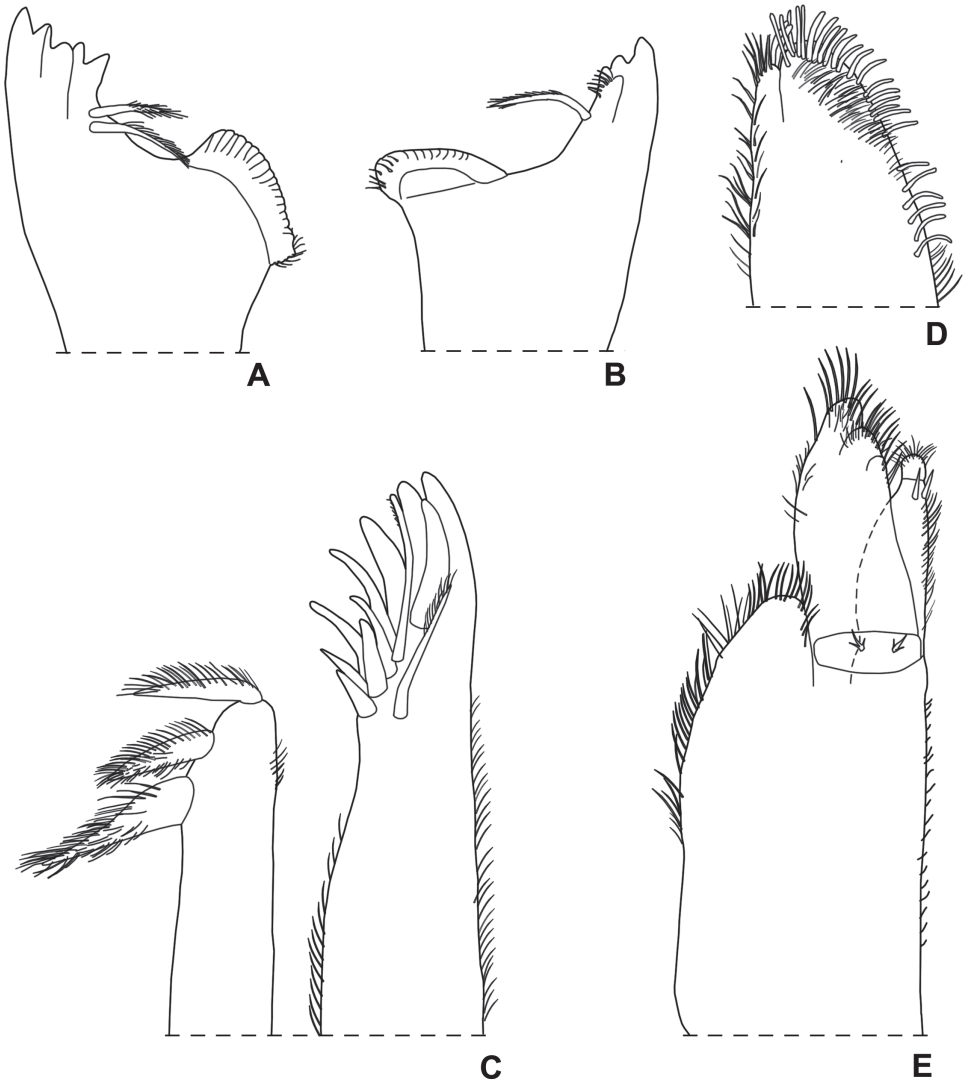

Figure 3. *Styloniscus manuvaka* sp. n., ♀ paratype: **A** left mandible **B** right mandible **C** maxillula **D** maxilla **E** maxilliped.

(Figs 1A, 2A). Vertex and pereon distinctly granulated with granulations arranged on three rows on pereonite 1 and two rows on pereonites 2-7; pleon and telson smooth. Dorsal surface with scale-setae as in Fig. 2B. Cephalon (Fig. 2C, D) with obtuse middle lobe slightly protruding frontwards compared with rounded lateral lobes; eye consisting of three ommatidia in a triangle. Pleonites 3-5 reduced with small posterior points. Telson (Fig. 2E) with concave sides and truncate apex. Antennula (Fig. 2F) with second article shorter than first and third; third article with 6 long aesthetascs at apex. Antenna (Fig. 2G) with flagellum as long as fifth article of peduncle; flagellum

cone-shaped, consisting of 5 articles with the second, third and fourth article bearing two aesthetascs each. Left mandible (Fig. 3A) with 2 penicils; right mandible (Fig. 3B) with 1 penicil. Maxillula (Fig. 3C) outer branch with 10 simple teeth and 2 long stalks; inner branch with 3 penicils. Maxilla (Fig. 3D) apically bilobate, inner lobe wider than outer lobe and bearing strong setae on its margin. Maxilliped (Fig. 3E) endite with a stout apical penicil; basal article of the palp with 2 setae. Pereopods 6 and 7 with a distinct water conducting system (Fig. 4B,C) on merus, carpus and propodus, and on basis, ischium and merus, respectively.

Male. Pereopod 1 (Fig. 4A) merus and carpus with a line of short scales on sternal margin. Pereopod 7 (Fig. 4C) ischium enlarged in the distal part, forming a flat rounded lobe with two short and stout setae on tergal margin, sternal margin almost straight; propodus with numerous long and thin setae on tergal margin. Genital papilla (Fig. 4D) with rounded and enlarged distal part. Pleopod 1 (Fig. 4D) exopodite triangular, as wide as long, with rounded posterior margin; endopodite with flagelliform distal segment, about twice as long as basal one and slightly enlarged at apex. Pleopod 2 (Fig. 4E) exopodite very short, rectangular, about twice wider than long; endopodite with distal segment about seven times longer than basal one, with tapering apical part slightly bent outwards, acute apex.

Etymology. The species name is a combination of two Rapanui terms, *manu* and *vaka*. *Manu* is "bug" and *vaka* is "canoe" or "boat"; when combined this translates to "canoe bug." Based upon the identification of this species, and a collembolan (*Lepidocyrtus olena* Christiansen & Bellinger, 1992) previously known from the Hawaiian Islands only, Wynne et al. (2014) suggested both of these animals may have been dispersed by the ancient Polynesians as they transported and transplanted cultivars (called "canoe plants"), such as banana, taro and sugar cane, throughout the South Pacific islands.

Remarks. At present the genus *Styloniscus* includes about 45 species distributed in the tropics and the southern hemisphere (Schmalfuss 2003; Nunomura 2007; Taiti 2014). The new species is characterized by the male pereopod 7 ischium enlarged in the distal part with a flat rounded lobe. A similar character is present also in a species from Omaio, North Island, New Zealand, identified by Vandel (1952) as *Styloniscus otakensis* (Chilton, 1901). The specimens redescribed and illustrated by Vandel certainly do not belong to *S. otakensis* according to the redescription of this species provided by Green (1971) on the basis of the type material studied by Chilton (1901) and on topotypic material (Dunedin, South Island). In fact, the male pereopod 7 ischium does not show any distinct lobe (compare fig. 31 in Green 1971 with fig. 37 in Vandel 1952), and the shapes of the male pleopod 1 exopodite and pleopod 2 endopodite are significantly different (compare figs 29 and 30 in Green 1971 with figs 38 and 39A in Vandel 1952). Thus, the specimens from Omaio must belong to a distinct species yet to be named. *Styloniscus manuvaka* sp. n. differs from *S. otakensis* sensu Vandel nec Chilton in having 6 instead of 5 aesthetascs at the apex of the antennula, 5 instead of 4 flagellar articles of the antenna, the male pereopod 7 ischium with two, instead of one, stout setae on the tergal margin, and the male pleopod 2 endopodite with a thicker distal part.

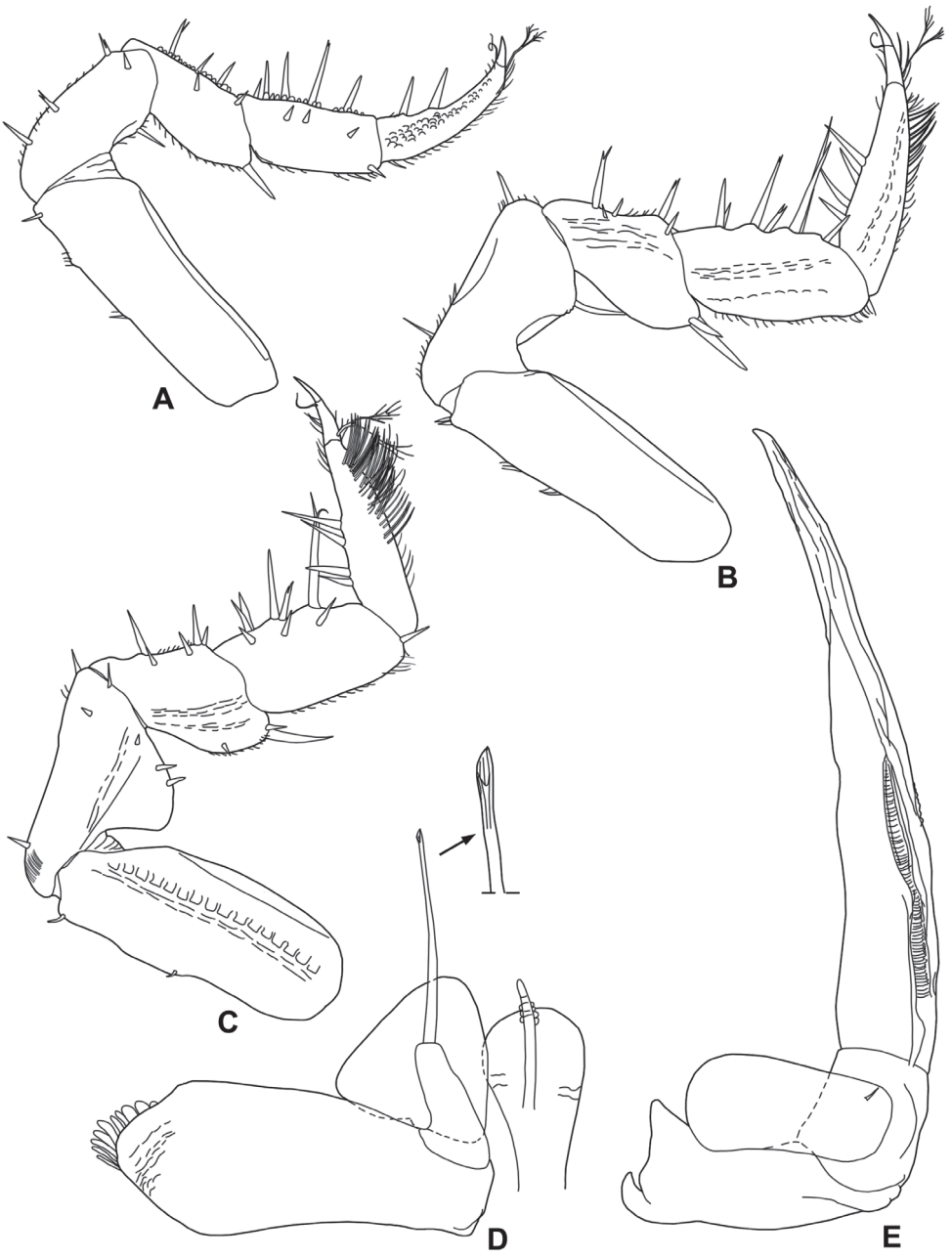

Figure 4. *Styloniscus manuvaka* sp. n., ♂ paratype: **A** pereopod 1 **B** pereopod 6 **C** pereopod 7 **D** genital papilla and pleopod 1 **E** pleopod 2.

On Rapa Nui, *Styloniscus manuvaka* sp. n. is presently restricted to the cave environment, but is not troglomorphic (cave-adapted). This animal was detected within the fern-moss gardens (entrance zone) of three caves, but also occurred within the

twilight and cave deep zones. This species was not detected during the surface sampling work conducted in 2009, nor has it been identified during previous invertebrate inventory work (e.g., Fuentes 1914, Olalquiaga 1946, Kuschel 1963, Campos and Peña 1973). The species also occurs on Rapa Iti, Bass Islands, where it is not restricted to the cave environment. This species is considered a Polynesian endemic and it might be present also on other Pacific islands.

Distribution. Presently known from Rapa Nui and Rapa Iti.

Family Philosciidae
Genus *Hawaiioscia* Schultz, 1973

***Hawaiioscia rapui* sp. n.**
http://zoobank.org/56E14D72-3CF5-4E39-A655-15659F01B67A
Figs 1B, 5–7

Hawaiioscia sp.; Wynne et al. 2014: 714, 716, fig. 2a.

Type material examined. Chile, Rapa Nui: 1 ♂ holotype, 2 ♀♀ paratypes (MNHN), Mahunga Hiva Hiva, Cave Q15-034, pitfall trap 5A (twilight zone) 12.VII.2009, leg. J.J. Wynne; 1 ♀ paratype (MZUF), 1 ♀ paratype (BPBM), same data, pitfall trap 7A (approx. deep zone); 1 ♂ Paratype (MZUF), Mahunga Hiva Hiva, Cave Q15-076/078, pitfall trap 2C (light zone), 4.VII.2009, leg. J.J. Wynne.

Description. Maximum length: ♂ and ♀ 7.5 mm. Dorsum light brown with the usual muscle spots (Fig. 1B). Body flat, ovoidal, with pleon narrower than pereon, outline as in Fig. 5A. Dorsal body surface finely granulated with small triangular scale-setae (Fig. 5B). Pereonites with no sulcus marginalis, gland pores absent. Noduli laterales (Fig. 5C, G) clearly visible, inserted on a small tubercle and disposed as follows: two on the cephalic vertex, one per side on pereonites 1-6 with that on the fourth pereonite much more distant from the lateral margin of the segment, and two per side on pereonite 7. Cephalon (Fig. 5D–F) with short triangular lateral lobes not protruding frontwards compared with the obtuse middle lobe; frontal and supra-antennal lines absent; eyes small, consisting of eight ommatidia. Pleon epimera reduced but with distinct posterior points (Fig. 5A,H). Telson (Fig. 5H) triangular, about twice as wide as long, with broadly rounded apex. Antennula (Fig. 5I) of 3 articles, second article slightly shorter than first and third; third article bearing two rows of 7 and 2 aesthetascs each, and 2 apical aesthetascs. Antenna (Fig. 6A) long and thin, reaching back rear margin of pereonite 6; flagellum as long as fifth peduncular article, first flagellar article distinctly longer than second and third, with two rows of 4 to 6 aesthetascs on each second and third article. Mandibles (Fig. 6B,C) with molar penicil semidichotomized, i.e. consisting of 3-4 setae on a common stem; left mandible with 2+1 and right mandible with 1+1 free penicils. Maxillula (Fig. 6D) outer branch with 5+6 teeth, all simple; inner branch with two stout subequal penicils. Maxilla (Fig. 6E) apically setose and bilobate with outer lobe wider

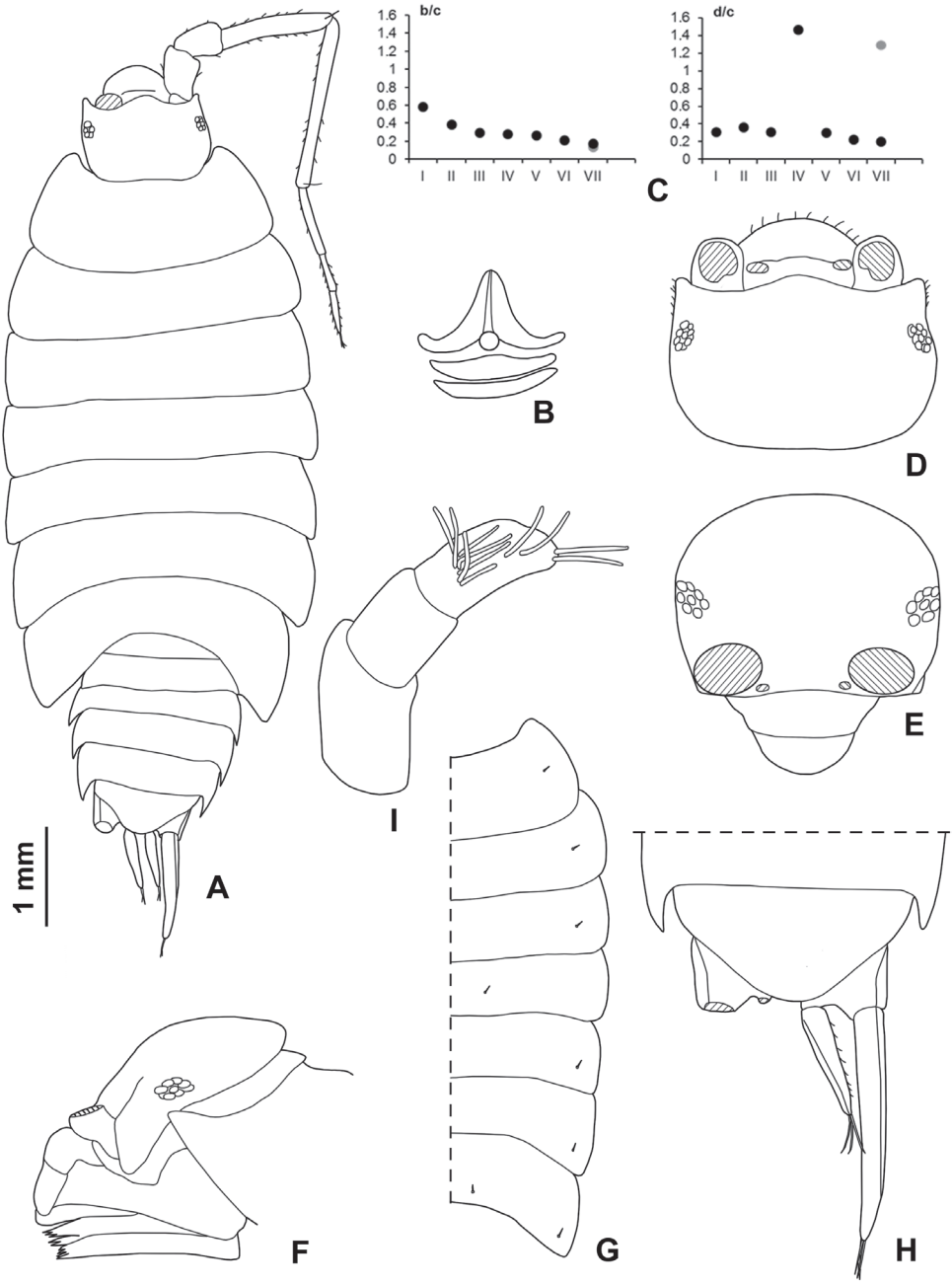

Figure 5. *Hawaiioscia rapui* sp. n., ♂ holotype: **A** adult specimen in dorsal view. ♀ paratype: **B** dorsal scale-seta **C** co-ordinates of noduli laterales **D** cephalon in dorsal view **E** cephalon in frontal view **F** cephalon in lateral view **G** pereonites with noduli laterales **H** pleonite 5, telson and uropods **I** antennula.

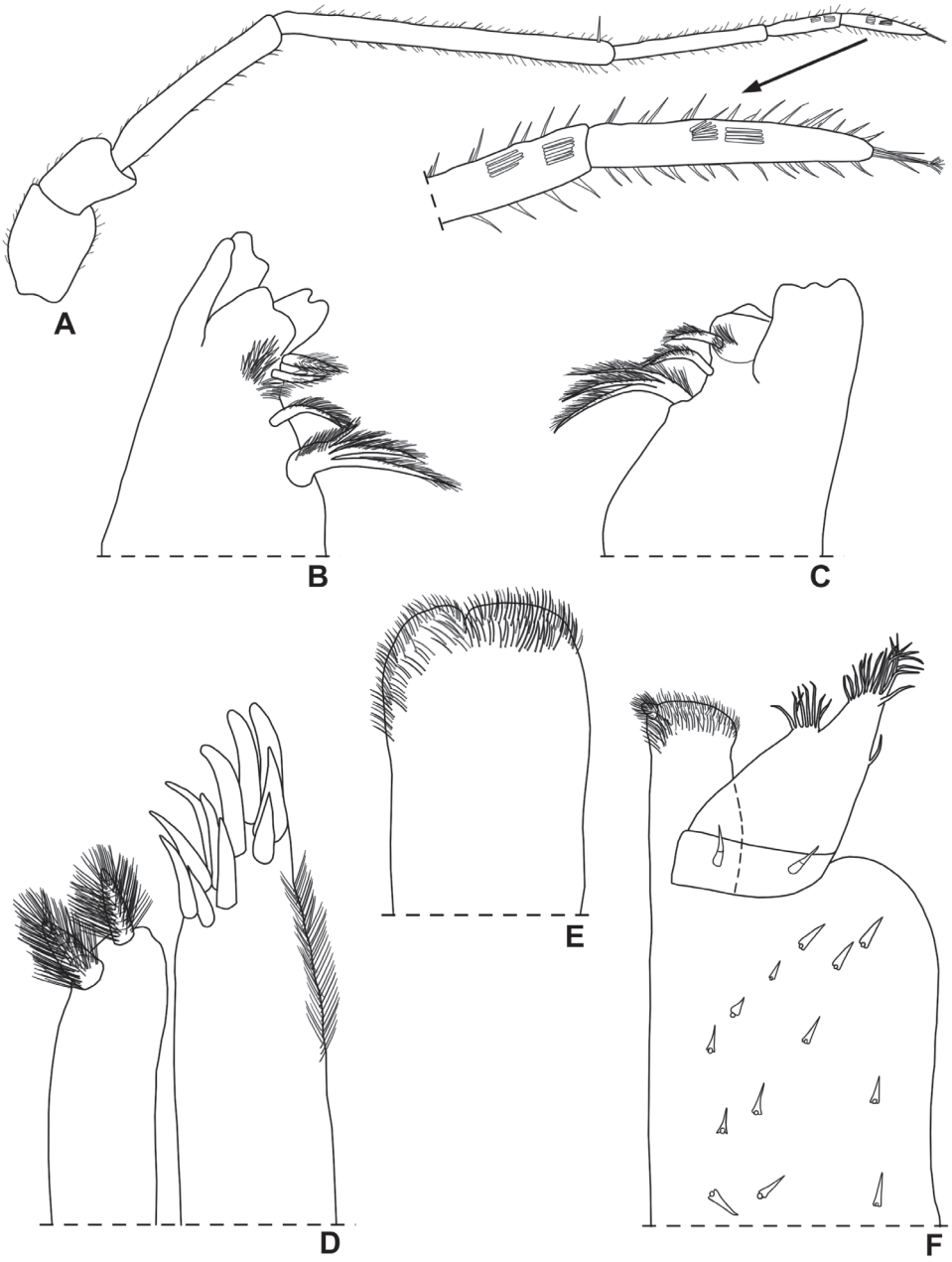

Figure 6. *Hawaiioscia rapui* sp. n., ♀ paratype: **A** antenna **B** left mandible **C** right mandible **D** maxillula **E** maxilla **F** maxilliped.

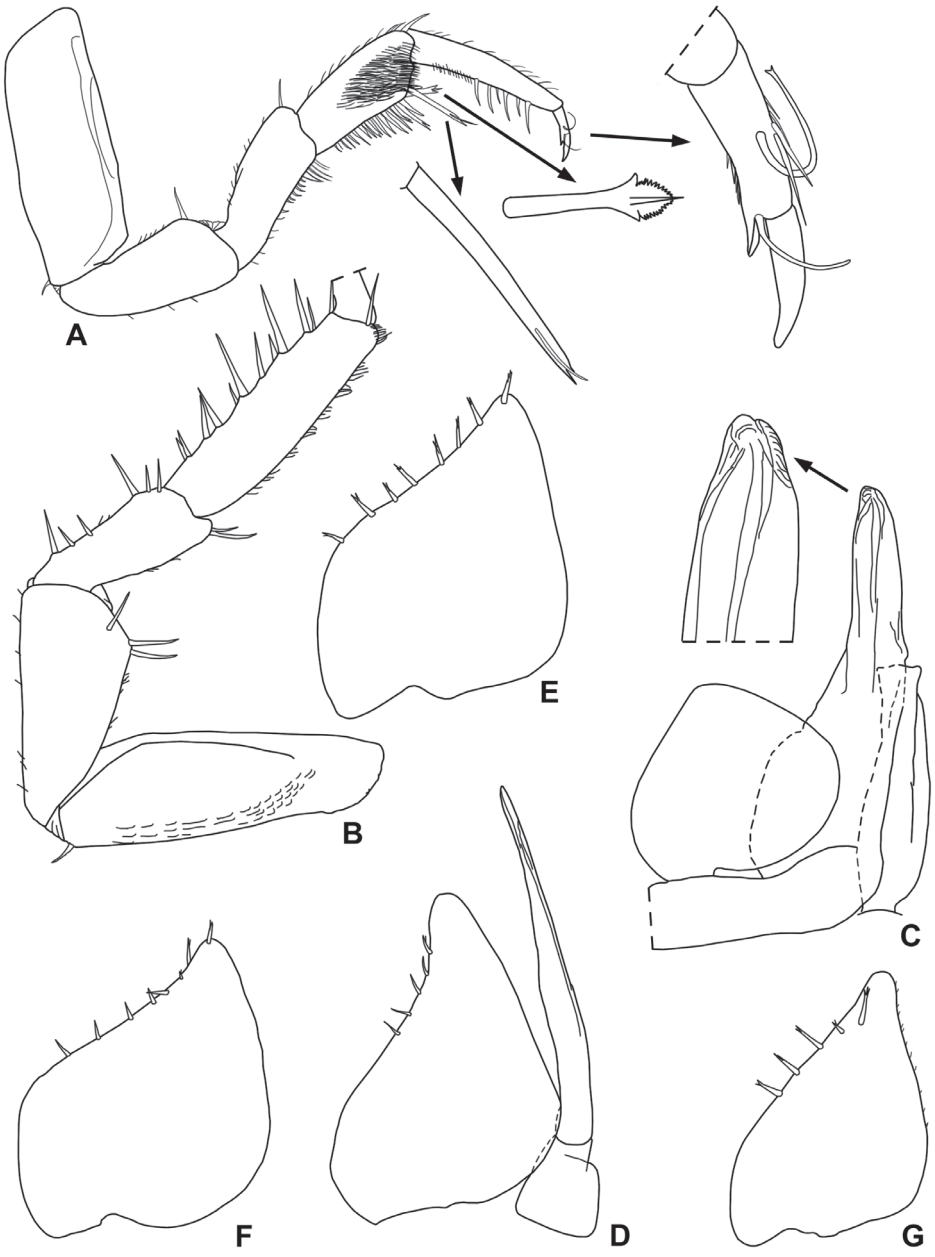

Figure 7. *Hawaiioscia rapui* sp. n., ♂ paratype: **A** pereopod 1 **B** pereopod 7 **C** genital papilla and pleopod 1 **D** pleopod 2 **E** pleopod 3 exopodite **F** pleopod 4 exopodite **G** pleopod 5 exopodite.

than inner one. Maxilliped (Fig. 6F) endite apically setose and bearing a large penicil at medial corner, proximal article of palp bearing 2 strong setae. Pereopods with elongated articles and flagelliform dactylar and ungual setae (Fig. 7A). Pleopodal exopodites with

no trace of respiratory structures. Uropod (Fig. 5H) protopod with a /\-shaped groove on outer margin; insertion of endopodite slightly proximal to that of exopodite.

Male. Pereopod 1 carpus with a brush of trifid spines on sternal margin (Fig. 7A). Pereopod 7 (Fig. 7B) with no peculiar modifications, ischium with sternal margin straight. Pleopod 1 (Fig. 7C) exopodite cordiform, with a broadly rounded apex; endopodite with thickset distal part, straight with rounded apex. Pleopod 2 (Fig. 7D) with exopodite triangular, shorter than endopodite and bearing 5 setae on ouer margin. Pleopods 3-5 exopodite as in Fig. 7E–G.

Etymology. The new species is named after Sergio Rapu Haoa, a humanitarian who has furthered cultural and archeological knowledge of Rapa Nui. Sergio was Rapa Nui's first governor of Rapanui descent and the first director and curator of Museo Antropológico P. Sebastián Englert on Rapa Nui. He is also a world-renowned Rapa Nui archaeologist and purveyor of Rapa Nui culture. He graciously provided logistical support to the second author and his research teams while on Rapa Nui.

Remarks. Prior to discovering this new species, the genus *Hawaiioscia* consisted of four troglomorphic species restricted to lava tube caves on the Hawaiian Islands (Schultz 1973; Taiti and Howarth 1997): *H. parvituberculata* Schultz, 1973 from Maui, *H. microphthalma* Taiti & Howarth, 1997 from Oʻahu, *H. paeninsulae* Taiti & Howarth, 1997 from Molokaʻi, and *H. rotundata* Taiti & Howarth, 1997 from Kauaʻi. No epigean species in this genus were previously known. The new species shows all the characters of the genus *Hawaiioscia* with the sole exception of the molar penicil of the mandible which is semidichotomized instead of simple as in all the others species from Hawaiʻi. Considering that all the most important characters (number and position of noduli laterales, maxillular teeth, penicil on maxillipedal endite, uropod and shape of male pleopod 1) are shared with all the other *Hawaiioscia* species, we include the new species in this genus.

Specimens from this new species were collected from both within the entrance zone of one cave and the twilight zone of another cave. It is important to note, this species does not have troglomorphic characteristics, such as body depigmentation or eye reduction as do other congeners within *Hawaiioscia*. However, as with *Styloniscus manuvaka* sp. n., this new species was not detected during the surface sampling effort, nor has it been previously identified by earlier entomological surveys of the island. Thus, we believe this animal to be restricted to cave environment on Rapa Nui.

Distribution. Presently endemic to Rapa Nui.

Family Platyarthridae
Genus *Trichorhina* Budde-Lund, 1908

***Trichorhina tomentosa* (Budde-Lund, 1893)**

Material examined. Chile, Rapa Nui: 1 ♀ (BPBM), Mahunga Hiva, Cave Q15-074, pitfall trap 1B (light zone), 30.VI.2009, leg. J.J. Wynne.

Distribution. Pantropical. Introduced to greenhouses worldwide.

Family Porcellionidae
Genus *Porcellionides* Miers, 1877

***Porcellionides pruinosus* (Brandt, 1833)**

Material examined. Chile, Rapa Nui: 2 ♂♂, 3 ♀♀ (AMNH 18360), Cannibal Cave, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 1 ♀ (BPBM), Mahunga Hiva Hiva, Cave, Q15-074, time search at 1A (light zone), 30.VI.2009, leg. J.J. Wynne; 1 ♂, 3 ♀♀ (BPBM), same location, leaf litter beneath skylight, (3rd entrance NW of main entrance; entrance zone), direct intuitive search, 2.VIII.2011, leg. J.J. Wynne; 1 ♀ (BPBM), Mahunga Hiva Hiva, Cave Q15-067, fern-moss garden (entrance zone), direct intuitive search, 10.VII.2009, leg. J.J. Wynne; 1 ♂ (BPBM), Mahunga Hiva Hiva, Cave Q15-070, fern-moss garden (entrance zone), direct intuitive search, 10.VII.2009, leg. J.J. Wynne.

Distribution. Cosmopolitan species of Mediterranean origin.

Genus *Porcellio* Latreille, 1804

***Porcellio scaber* Latreille, 1804**

Porcellio scaber; Fuentes 1914: 315; Wynne et al. 2014: 716.

Material examined. Chile, Rapa Nui: 1 ♀ (AMNH 18362), VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 1 ♀ (AMNH 18363), Maunga Tangaroa, 20.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 3 ♀♀ (AMNH 18365), Ana te Pahu, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 13 ♀♀ (AMNH 18364), Poike region, 25.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 1 ♂ (BPBM), Mahunga Hiva Hiva, surface in front of Cave Q15-038, opportunistic collection (eastern-most collapse pit, southern extent near cave entrance), 18.VIII.2008, J.J. Wynne; 1 ♂ (BPBM), Mahunga Hiva Hiva, surface grid 2, 27°06'41.3"S, 109°25'09.2"W, pitfall trap 21, 10.VII.2009, leg. J.J. Wynne; 1 juv. (BPBM), Mahunga Hiva Hiva, surface in front of Cave Q15-038, timed search at 1C (eastern-most collapse pit on southern extent near cave entrance), 20.VIII.2008, leg. J.J. Wynne; 3 ♂♂ (BPBM), Cave Q15-038, fern-moss garden (entrance zone), direct intuitive search, 4.XII.2008, leg. J.J. Wynne; 1 ♂, 2 ♀♀ (BPBM), Mahunga Hiva Hiva, Cave Q15-076/078, opportunistic collection, 4.VII.2009, leg. J.J. Wynne; 1 ♂, 2 ♀♀ (BPBM), Mahunga Hiva Hiva, Cave Q15-070, fern-moss garden (entrance zone), direct intuitive search, 13.VII.2009, leg. J.J. Wynne; 2 ♂♂, 1 ♀ (BPBM), Mahunga Hiva Hiva, Cave Q15-074, skylight entrance (1st entrance NW of main entrance; entrance zone), opportunistic collection, 3.VII.2009, leg. J.J. Wynne; 1 juv. (BPBM), Ma-

hunga Hiva Hiva, Cave Q15-067, fern-moss garden (entrance zone), direct intuitive search, 10.VII.2009, leg. J.J. Wynne; 1 ♀ (BPBM), Mahunga Hiva Hiva, Cave Q15-127, entrance zone, pitfall trap 1A, 5.VII.2009, leg. J.J. Wynne; 1 ♂ (BPBM), same data, pitfall traps 1B; 1 ♂, 1 ♀ (BPBM), Mahunga Hiva Hiva, surface grid 1, 27°06'53.1"S, 109°24'20.3"W, pitfall trap 3, 10.VII.2009, leg. J.J. Wynne; 1 ♂, 1 ♀ (BPBM), same location, pitfall trap 10, 10.VII.2009, leg. J.J. Wynne; 2 ♂♂, 5 ♀♀ (BPBM), same location, pitfall trap 12, 10.VII.2009, leg. J.J. Wynne.

Distribution. Cosmopolitan species of western European origin.

Porcellio laevis Latreille, 1804

Material examined. Chile, Rapa Nui: 2 ♂♂ (AMNH 18362), VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 1 juv. (AMNH 18363), Maunga Tangaroa, 20.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 1 ♂, 1 ♀ (AMNH 18365), Cave Q15-074, location within cave not reported, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 7 ♀♀ (AMNH 18361), La Pérouse Bay, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez.

Distribution. Cosmopolitan species of Mediterranean origin.

Family Armadillidiidae
Genus *Armadillidium* Brandt, 1831

Armadillidium vulgare (Latreille, 1804)

Armadillidium vulgare; Fuentes, 1914: 315.

Material examined. Chile, Rapa Nui: 1 ♂ (AMNH 18362), VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 6 ♂♂, 5 ♀♀ (AMNH 18365), Cave Q15-074, location within cave not reported, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 3 ♂♂, 9 ♀♀ (AMNH 18366), Hotel Hanga Roa, Hanga Roa, 21.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 2 ♂♂, 2 ♀♀ (AMNH 18364), Poike region, 25.VIII.1999, leg. C. Boyko, J. Tanacredi, S. Reanier, H. Tonnemacher and S. Lopez; 1 ♂, 1 ♀ (BPBM), Mahunga Hiva Hiva, surface in front of Cave Q15-038, timed search at 1B (eastern-most collapse pit, southern extent near cave entrance), 20.VIII.2008, leg. J.J. Wynne; 1 ♀ (BPBM), Cave Q15-038, fern-moss garden (entrance zone), direct intuitive search, 21.XIII.2008, leg. J.J. Wynne; 2 ♀♀ (BPBM), Mahunga Hiva Hiva, surface grid 2, 27°06'41.3"S, 109°25'09.2"W, pitfall trap 23, 10.VII.2009, leg. J.J. Wynne; 1 ♂ (BPBM), surface grid 1, 27°06'53.1"S, 109°24'20.3"W, pitfall trap 17, 10.VII.2009, leg. J.J. Wynne; 3 ♀♀ (BPBM), same

location, pitfall trap 19, 10.VII.2009, leg. J.J. Wynne; 1 ♂, 3 ♀♀ (BPBM), same location, pitfall trap 4, 10.VII.2009, leg. J.J. Wynne.

Distribution. Cosmopolitan species of Mediterranean origin.

Family Armadillidae
Genus *Venezillo* Verhoeff, 1928

***Venezillo parvus* (Budde-Lund, 1885)**

Material examined. Chile, Rapa Nui: 2 ♂♂ (BPBM), Mahunga Hiva Hiva, surface grid 2, 27°06'41.3"S, 109°25'09.2"W, pitfall trap 39, 10.VII.2009, leg. J.J. Wynne; 1 ♂ (BPBM), same location, pitfall trap 32, 10.VII.2009, leg. J.J. Wynne.

Distribution. Widespread in tropical and subtropical regions. It has been introduced to European greenhouses. For diagnostic figures of this species see Schmidt (2003).

Key to species of terrestrial isopods from Rapa Nui

1	Antennal flagellum with >10 articles; eye with >100 ommatidia	***Ligia exotica***
–	Antennal flagellum with <6 articles, eye with <30 ommatidia	**2**
2	Antennal flagellum of 5 articles	***Styloniscus manuvaka***
–	Antennal flagellum of 3 or 2 articles	**3**
3	Antennal flagellum of 3 articles	***Hawaiioscia rapui***
–	Antennal flagellum of 2 articles	**4**
4	Body depigmented; eye consisting of a single ommatidium	***Trichorhina tomentosa***
–	Body pigmented; eye consisting of several ommatidia	**5**
5	Body slightly convex, unable to roll up into a ball	**6**
–	Body strongly convex, able to roll up into a perfect ball	**8**
6	Cephalon with a V-shaped suprantennal line; pereonite 1 with posterior margin straight and posterior corners rounded	***Porcellionides pruinosus***
–	Cephalon without suprantennal line; pereonite 1 with posterior margin more or less concave at sides and posterior corners right-angled or acute	7
7	Dorsal body surface smooth	***Porcellio laevis***
–	Dorsal body surface distinctly granulated	***Porcellio scaber***
8	Cephalon with a triangular frontal scutellum; telson trapezoidal; uropod exopodite flattened, filling the gap between telson and pleonite 5	***Armadillidium vulgare***
–	Cephalon with no frontal scutellum; telson hour-glass shaped; uropod protopodite flattened, filling the gap between telson and pleonite 5	***Venezillo parvus***

Discussion

Nine species of terrestrial isopods are known from Rapa Nui: *Ligia exotica, Styloniscus manuvaka* sp. n., *Hawaiioscia rapui* sp. n., *Trichorhina tomentosa*, *Porcellionides pruinosus*, *Porcellio laevis*, *P. scaber*, *Armadillidium vulgare*, and *Venezillo parvus*.

Only one species (*Ligia exotica*) is littoral, halophilic, and widely distributed along coastal habitats in the tropics. We have not examined any specimens belonging to this species and its identification needs to be confirmed. Littoral habitats have not been adequately sampled on Rapa Nui and other littoral species may also be present on the island. Two species (*Trichorhina tomentosa* and *Venezillo parvus*) have a wide distribution in the tropics, and four species of European or Mediterranean origin (*Porcellionides pruinosus*, *Porcellio laevis*, *P. scaber*, and *Armadillidium vulgare*) are now cosmopolitan. All of these species were introduced to Rapa Nui due to human activities. *Styloniscus manuvaka* sp. n. and *Hawaiioscia rapui* sp. n. are Polynesian and Rapa Nui endemics, respectively.

Given that few native arthropod species remain on Rapa Nui (Wynne et al. 2014), the two new isopod species are a significant contribution to the island's natural history. Together with the other eight endemics described by Bernard et al. (2015), Mockford and Wynne (2013) and Jordana and Baquero (2008), these disturbance relicts have persisted despite several hundred years of extreme environmental change and interactions with non-native species (Wynne et al. 2014).

Despite their persistence, these endemic species are considered imperiled (Wynne et al. 2014). *S. manuvaka* and *H. rapui* may be operating under extinction debts (Triantis et al. 2010). This may occur once a population has become isolated following a significant environmental perturbation, such as habitat loss or fragmentation (Tilman et al. 1994). Habitat loss has occurred dramatically and at an island-wide scale on Rapa Nui. Both *S. manuvaka* and *H. rapui* were detected in low numbers. Neither of these species were detected during earlier inventory work (see Fuentes 1914, Olalquiaga 1946, Kuschel 1963, Campos and Peña 1973) or our surface sampling effort.

Further, the combined effects of global climate change and interactions with non-native species may further threaten the persistence of these endemic isopods. Competition with non-native species has been identified as threatening the persistence of surface-dwelling endemic arthropods on other island ecosystems (see Chown et al. 2007, Fordham and Brook 2010, Vitousek et al. 1997). Increased drought conditions are predicted for the sub-tropics (IPCC 2013) and other Polynesian islands (Chu et al. 2010). We also know non-native species represent the majority of known arthropods on Rapa Nui. For example, of the seven known non-native isopod species, the cosmopolitan *P. scaber* was detected in the greatest numbers in both surface sampling and within caves (Wynne et al. 2014). Additionally, *P. scaber* is a well-established non-native species being first detected by Fuentes (1914). In Hawai'i, *P. scaber* is considered to be an invasive species and one of the most damaging non-native arthropods in the native ecosystems (Howarth et al. 2001).

Conservation and management of these endemic terrestrial isopods (as well as the other endemic species) and their habitats should be a high priority for the Rapanui community, policy makers and resource managers. Given the concerns associated with global climate change and non-native invasive species, a captive breeding program of these new species is recommended. Captive breeding of isopods is relatively easy and inexpensive (Sutton 1972). Such a program may be developed in collaboration with CONAF, Museo Antropológico P. Sebastián Englert de Rapa Nui and potentially secondary school classrooms on the island. By captively breeding these animals in a variety of locations, their long-term persistence may be somewhat safeguarded, and will facilitate the establishment of viable populations for future reintroduction efforts. Additionally, this will provide an opportunity for researchers to obtain information associated with the life history characteristics of these endemic species. Also, once large captive populations are established, experiments examining competition with the non-native isopods may be performed.

Northup and Welbourn (1997) proposed that moss garden habitats in New Mexico lava tube caves may serve as a source habitat for arthropods colonizing cave deep zones. Fern-moss gardens within Rapa Nui caves may provide this same function. All known congeners of *H. rapui* sp. n. are cave-adapted isopods from the Hawaiian Islands. *H. rapui* sp. n. was detected within both entrance and twilight zones. If this species persists, it is possible parapatric speciation may occur as has been suggested for other *Hawaiioscia* species from the Hawaiian Islands (Rivera et al. 2002).

Finally, we know little concerning the distributions of these endemic isopods. We recommend additional surveys be conducted in other caves on the island, as well as in other habitats likely to support terrestrial isopods (and endemic arthropods, in general). This final step will provide resource managers with the ability to better characterize endemic isopod habitat, and to further improve our understanding of the distribution of these animals on Rapa Nui.

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First record and redescription of the terrestrial isopod *Hemilepistoides messerianus* Borutzky, 1945 (Isopoda, Oniscidea) from Iran

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Abstract

In the present study, *Hemilepistoides messerianus* Borutzky, 1945 is reported from Iran for the first time. This species is redescribed and diagnostic characters of both males and females are illustrated. This species is characterized by the tuberculation of all parts of the dorsal surface of the body and the male pleopod endopodite I with a triangular lobe at apex. A map with the distribution of species is presented.

Keywords

Oniscidea, *Hemilepistoides messerianus*, redescription, Turkmenistan, Iran

Introduction

Among a dozen species of terrestrial isopods from Turkmenia [now Turkmenistan], Borutzky (1945) established the new genus and species *Hemilepistoides messerianus* for two female specimens collected from Ashgabat region. No further study on this taxon has been published since. The type material was deposited in the Zoological Museum of Moscow State University (ZMMU) (Borutzky 1972). Re-examination of type material of terrestrial isopods in ZMMU revealed that the type specimens of *H. messerianus* are presumably lost.

In a survey on terrestrial isopods of northern Iran, many specimens belonging to the genus *Hemilepistoides* were found. Comparison of female specimens collected from Iran with description and illustrations presented by Borutzky (1945) for *H. messerianus* revealed no marked difference between them, convincing that they belong to the same species.

The aim of the present study is to redescribe *H. messerianus* on the basis of both female and male specimens from Iran.

Material and methods

All material was collected by the author in northern Iran. The specimens were collected by hand and preserved in 96% ethanol. The isopods were dissected and body parts were mounted in micropreparations using Euparal (Carl Roth, Karlsruhe). Drawings were made using a drawing tube fitted on a Nikon Y-IDT compound microscope. Micrographs were taken using a Hitachi S-2460N SEM.

Some material of the present study was deposited in the Zoological Museum, University of Tehran (ZUTC) and Iranian Research Institute of Plant Protection (IRIPP), and the others were kept in the personal collection of the author (PCGMK). A map with sampling localities for *H. messerianus* in Iran along with the type locality is presented (Fig. 1).

Taxonomy

Order Isopoda Latreille, 1817
Suborder Oniscidea Latreille, 1802
Family Agnaridae Schmidt, 2003

Genus *Hemilepistoides* Borutzky, 1945

Type species. *Hemilepistoides messerianus* Borutzky, 1945

Diagnosis. Body narrow and elongated, dorsal parts bearing rounded tubercles. Head with developed lateral lobes; frons with a distinct incision in the middle; no supra-antennal line. Antenna with flagellum of two articles, proximal segment longer than distal one. Pereon epimera I with rounded posterolateral margin. Pleotelson triangular with rounded apex and slightly concave sides. Male pereopods I-II with brushes of setae on sternal margin of merus and carpus; pereopod VII with sinuate sternal margin. Pleopod exopodites I-V with monospiracular covered lungs. Runner type according to the eco-morphological classification proposed by Schmalfuss (1984).

Remarks. Among the members of the family Agnaridae, the genus *Hemilepistoides* is similar to the members of the subgenus *Hemilepistus* (*Desertellio*), from which it

Figure 1. Sampling localities of *Hemilepistoides messerianus* in Iran and position of the type locality (asterisk) in Turkmenistan. The numbers refer to localities listed in the material examined.

differs in possessing tubercles also on all posterior parts of the body. The genus *Hemilepistoides* is a monotypical taxon with *H. messerianus* distributed in southern Turkmenistan and northern Iran.

Hemilepistoides messerianus Borutzky, 1945

Material examined. Semnan, [1] Shahrood, Kalate-Khij, 36°40.1'N, 55°18.7'E, 6 May 2008, two females (ZUTC 5327); the same data as before, two males and two females (PCGMK 1178); [2] Khorasan Shomali, 5 km W of Shirvan, 37°25.1'N, 57°52.7'E, 7 May 2008, one male and one female (ZUTC 5328); same data, two males and nine females (PCGMK 1182); [3] Golestan, S of Gonbade-Kavoos, 37°13.3'N, 55°09.8'E, 10 September 2008, two males and one female (PCGMK 1308); [4] N of Gonbade-Kavoos, 37°16.0'N, 55°10.0'E, 10 September 2008, two males and one females (ZUTC 5329); same data, ten males and ten females (PCGMK 1309); [5] 7

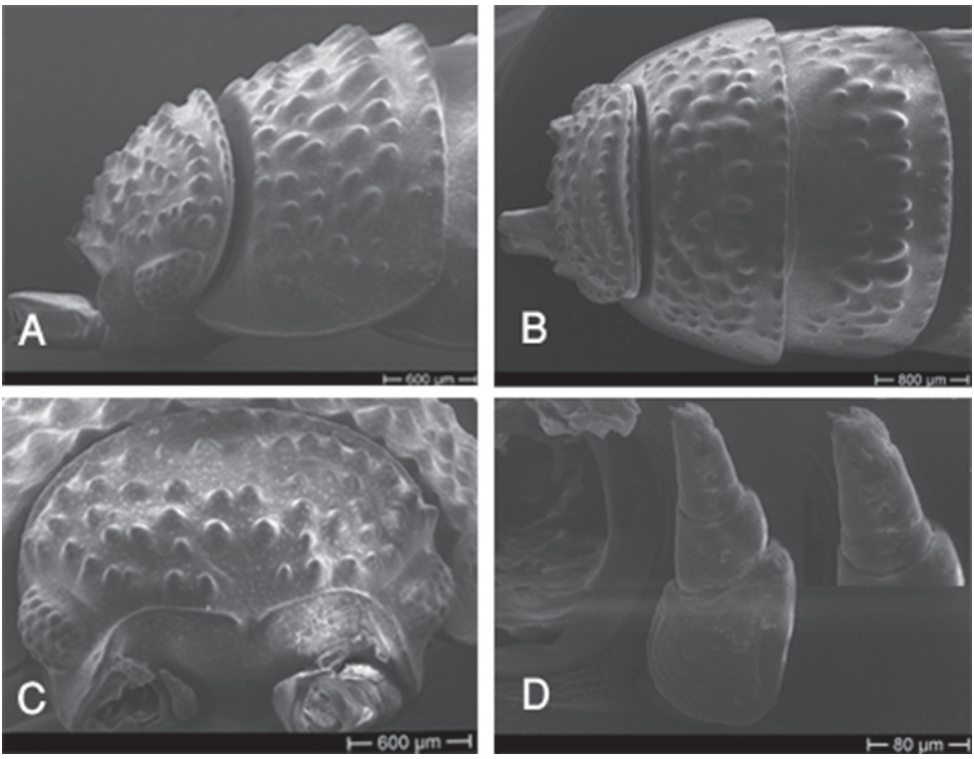

Figure 2. *Hemilepistoides messerianus*, female, from [2]. **A** Head and first pereonite, lateral view **B** head and first pereonite, dorsal view **C** head, frontal view **D** antennule and enlarged distal article.

km E of Maraveh-Tappeh, 37°54.6'N, 56°02.2'E, 2 August 2014, one female (IRIPP Iso-1052); [6] 10 km N of Gomishan, 37°54.6'N, 56°02.2'E, 4 August 2014, one male, two females (IRIPP Iso-1053); [7] Mazandaran, 3 km E of Behshahr, 36°22.5'N, 53°38.8'E, 4 August 2014, one female (IRIPP Iso-1054).

Diagnosis. Cephalothorax with rounded lateral lobes, frons with incision in the middle; dorsal parts of the body bearing rounded tubercles. Male pleopod endopdite I straight, with a leaf-like lobe at apex.

Redescription. Maximum length of both male and female: 15 mm. Body elongated, three times as long as wide. Color: cephalothorax and pleon dark; pereon, pale brown with a median longitudinal dark band or thoroughly dark with pale epimera.

Cephalothorax with developed rounded lateral lobes, vertex with several rounded tubercles of almost the same size; frontal line sinuous in frontal view, with a distinct incision in the middle; no supraantennal line (Fig. 2C); eyes with 20–24 ommatidia. Antenna long, reaching posterior margin of the second pereon-tergite; flagellum slightly shorter than fifth article of peduncle, with two articles, first article about twice as long as second (Fig. 3B). Antennule of three articles with a tuft of short aesthetascs at apex (Fig. 2D). Pereon-tergites with rounded tubercles, arranged in several rows on the first

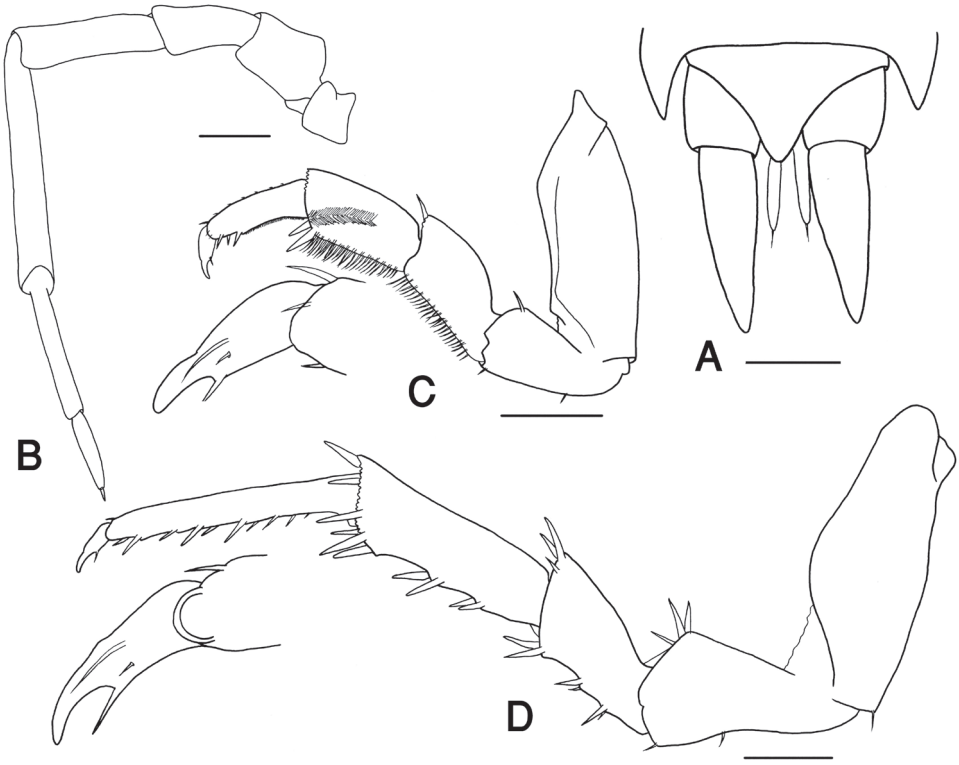

Figure 3. *Hemilepistoides messerianus*, male, from [2]. **A** Telson and uropods **B** antenna **C** pereopod 1 **D** pereopod 7. scales: 0.5 mm.

tergite, median tubercles larger than lateral ones, decreasing in number on posterior tergites. Pereon-tergite I with rounded posterolateral margins (Fig. 2A–B).

Pleon slightly narrower than pereon, each pleon-tergite with a row of faint tubercles on the posterior margin. Pleotelson triangular, with slightly concave sides and rounded apex. Uropod exopodites conical, about 1.5 times as long as pleotelson (Fig. 3A). Pleopod exopodites I-V with monospiracular covered lungs (Fig. 4B–I).

Male: Pereopod I merus and carpus with brushes of setae on sternal margin; propodus narrow and long, proximal part of sternal margin with dense small scales, distal part bearing strong setae (Fig. 3C). Pereopod II merus and carpus with brushes of setae on ventral margin. Pereopod VII ischium with sinuate sternal margin; merus and carpus equipped with strong setae; propodus narrow and long (Fig. 3D). Pleopod endopodite I straight, apex with a triangular lobe, equipped with a row of small setae on inner margin (Fig. 4A). Pleopod exopodite I with a short rounded hind lobe; inner margin with a row of small setae (Fig. 4B). Pleopod exopodite II triangular, with a row of setae on outer margin; endopodite slightly longer than exopodite (Fig. 4C). Pleopod exopodites III-V as in Fig. 4D–F.

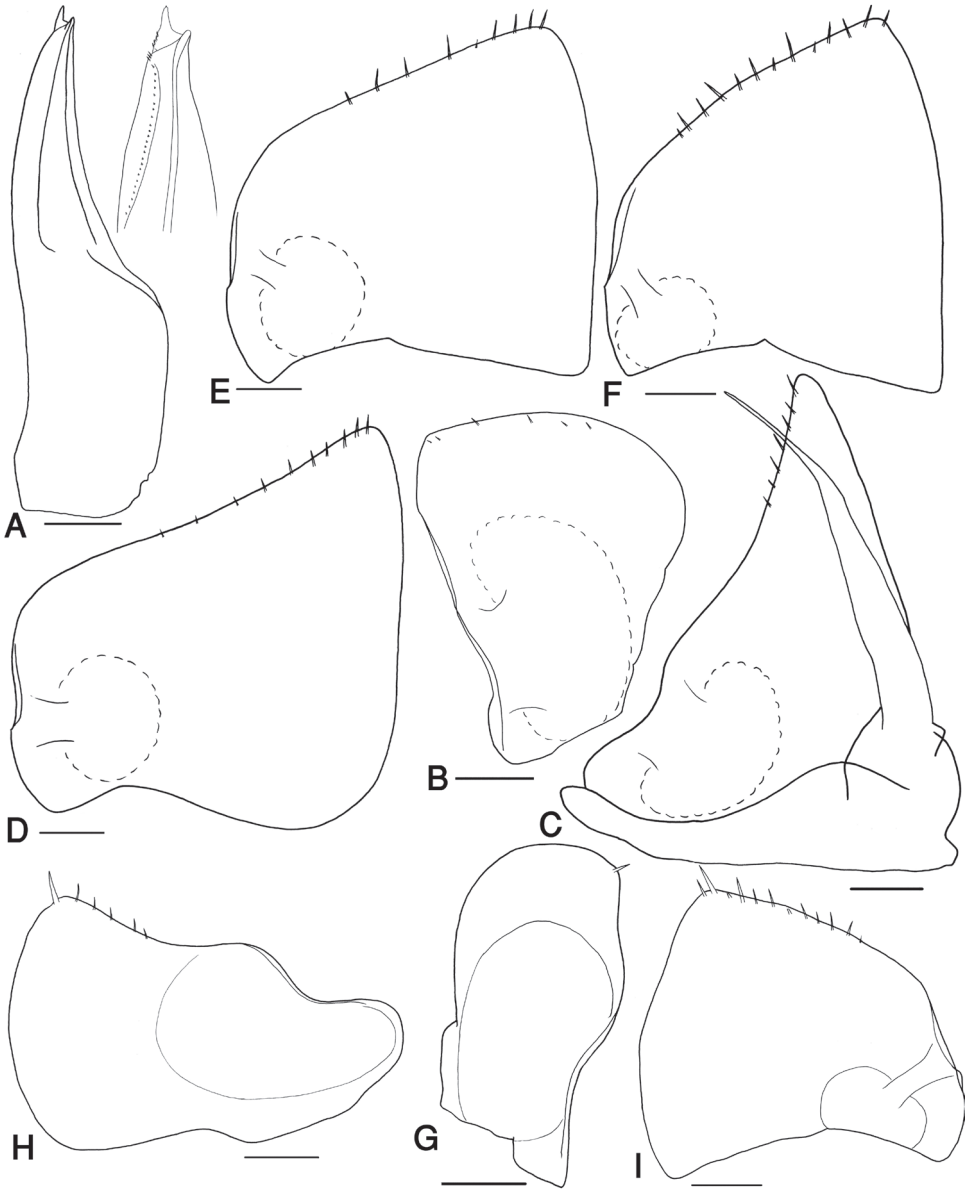

Figure 4. *Hemilepistoides messerianus*, **A–F** male from [3] **G–I** female from [6]. **A** pleopod endopodite I **B** pleopod exopodite I **C** pleopod II **D** pleopod exopodite III **E** pleopod exopodite IV **F** pleopod exopodite V **G** pleopod exopodite I **H** pleopod exopodite II **I** pleopod exopodite V. Scales: 0.2 mm.

Female: Pereopod I merus and carpus without brushes of setae on sternal margin; pereopod VII ischium with straight sternal margin. Pleopod exopodite I with a rounded hind lobe bearing a single spine seta at apex (Fig. 4G). Pleopod exopodite II with two rounded lobes on posterior margin, inner lobe longer than the outer one; a

row of setae on posterior margin of inner lobe (Fig. 4H). Pleopod exopodite V as in Fig. 4I, very similar to that of males.

Remarks. Male characteristics are vital for species identification in most terrestrial isopods (Schmidt 2002). *Hemilepistoides messerianus* was described on the basis of female specimens; therefore the identification of the specimens found in Iran with this species might be problematic. Since this species is relatively broadly distributed in northern Iran, the type locality (Ashqabat, Turkmenistan) is not very far from the geographical range of the species in Iran (Fig. 1), and the female characteristics of the Iranian specimens are similar to those of type material described and illustrated by Borutzky (1945), it seems reasonable that they belong to the same species.

This species is distinguished by the shape of the male pleopod endopodite I, with apex bearing a triangular lobe.

Distribution. Southern Turkmenistan; northern Iran.

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The agnarid terrestrial isopods (Isopoda, Oniscidea, Agnaridae) of the province of Qazvin, Iran, with a description of a new species

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Abstract

Six species of terrestrial isopods from the province of Qazvin, central Iran, are recorded. Three species, *Hemilepistus klugii* (Brandt, 1833), *Protracheoniscus ehsani* Kashani, 2014 and *Mongoloniscus persicus* Kashani, 2014, were previously reported from the province. *Hemilepistus elongatus* Budde-Lund, 1885 and *Protracheoniscus major* (Dollfus, 1903) are recorded for the first time, and one species, *Protracheoniscus sarii* **sp. n.**, is described as new. The diagnostic characters of the new species are figured.

Keywords

Oniscidea, Agnaridae, new species, Qazvin, Iran

Introduction

Several contributions on the terrestrial isopod fauna of Iran have recently been published (Khalaji-Pirbalouti and Wägele 2010; Kashani et al. 2010, 2011, 2013; Kashani and Sari 2012; Kashani 2014a, 2014b); however, the knowledge on this taxon remains relatively poor. During a survey of terrestrial isopods of the province of Qazvin, a dozen species of terrestrial isopods were collected. In the present study the species belonging to the family Agnaridae are investigated.

The family Agnaridae is characterized by possessing monospiracular covered lungs in all five pleopod-exopodites (Schmidt 2003, 2008). Distributed in the temperate and subtropical zones of Eurasia and northern Africa, the members of this family prefer habitats with low humidity (Schmidt 2003). According to the world catalogue of terrestrial isopods by Schmalfuss (2003), the family includes 15 genera, the validity of some of which is questionable (Ferrara and Taiti 1988). Up to date, three genera of Agnaridae, i.e. *Hemilepistus* Budde-Lund, 1879, *Protracheoniscus* Verhoeff, 1917 and *Mongloniscus* Verhoeff, 1930, were reported from Iran (Kashani et al. 2010; Kashani 2014a, 2014b) and three agnarid species, namely *Hemilepistus klugii* (Brandt, 1833), *Protracheoniscus ehsani* Kashani, 2014 and *Mongloniscus persicus* Kashani, 2014, were recorded from the province of Qazvin (Kashani et al. 2010; Kashani 2014b). Here, we report the occurrence of three more specie, one of which is new to science.

Material and methods

The material of the present study was collected throughout the province of Qazvin. The specimens were collected by hand and preserved in 96% ethanol. Some of the specimens were dissected and the body parts were slide-mounted in Euparal (Carl Roth, Karlsruhe). Drawings were made using a camera lucida fitted on a SaIran ZSM-100 dissecting stereomicroscope and on a Nikon Y-IDT compound microscope. The specimens, including the type material of the newly described species have been deposited in the personal collection of the third author (PCGMK), the Zoological Museum, University of Tehran (ZUTC), and the Iranian Research Institute of Plant Protection, Tehran (IRIPP).

Taxonomy

Order Isopoda Latreille, 1817
Suborder Oniscidea Latreille, 1802
Family Agnaridae Schmidt, 2003
Genus *Hemilepistus* Budde-Lund, 1879

***Hemilepistus klugii* (Brandt, 1833)**

Material examined. Qazvin, 36°03.9'N, 50°03.6'E, 15 June 2008, leg. G.M. Kashani, two males and two females (ZUTC Iso.1059); Abgarm, Ardalan village, 35°53.6'N, 48°54.7'E, 21 June 2008, leg. G.M. Kashani, four males and six females (ZUTC Iso.1060); Boin-Zahra, Ebrahim-abad village, 10 October 2004, leg. M. Hakimzadeh, one male (PCGMK 1123); 5 km to Sagzabad, 35°46.4'N, 50°01.4'E, 18 June 2013, leg. G.M. Kashani & B. Eshaghi, one male (PCGMK 1656); Takestan to Zein-abad, 35°51.9'N, 49°52.5'E, 18 June 2013, leg. G.M. Kashani & B. Eshaghi, one male and two females (PCGMK 1660); Abgarm, 35°48.7'N, 49°08.0'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, one male (PCGMK 1705a).

Remarks. Kashani et al. (2010) reported the presence of *Hemilepistus klugii* in central parts of Iran, including the province of Qazvin. Here, more localities are presented for the species. This species occurs in semi-arid habitats of the province.

Distribution. Azerbaidjan and central Iran.

Hemilepistus elongatus Budde-Lund, 1885

Material examined. Takestan to Shal, 35°54.1'N, 49°48.0'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, five males and two females (PCGMK 1661); Esfarvarin to Takestan, 35°58.0'N, 49°43.1'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1664); Abyek to Gheshlagh, 36°01.3'N, 50°30.3'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one male and two females (PCGMK 1680).

Remarks. Despite the broad distribution of *H. elongatus* in Iran (Kashani and Sari 2012), this is the first time this species is reported from the province of Qazvin.

Distribution. "Transcaucasus"; easternmost Turkey: Ararat; Turkmenia; Iran.

Genus *Mongoloniscus* Verhoeff, 1930

Mongoloniscus persicus Kashani, 2014

Material examined. Boin Zahra, 30 June 2008, leg. G.M. Kashani, one male (PCGMK1627); Nikouieh, 36°16.2'N, 49°31.6'E, 11 September 2013, one male (PCGMK 1696); Abgarm, Chehel-Cheshmeh village, 35°46.6'N, 49°18.5'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, four males and two females (PCGMK 1706); Qazvin to Nikouieh, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1768); Nikouieh, Charandagh village, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, three males and seven females (PCGMK 1770); Nikouieh to Manjil, 5 June 2014, leg. G.M. Kashani & B. Eshaghi, seven males and twelve females (PCGMK 1774b).

Remarks. The presence of *Mongoloniscus persicus* in western Iran, including the province of Qazvin, was formerly reported by Kashani (2014b). Herein, only the sampling localities for the province are presented.

Distribution. Western Iran.

Genus *Protracheoniscus* Verhoeff, 1917

Protracheoniscus major (Dollfus, 1903)

Material examined. Saveh to Boin-Zahra, Seyd-abad village, 35°20.0'N, 50°13.0'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, two males (PCGMK 1653); Boin-Zahra to Segzabad, 35°46.4'N, 40°03.0'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, four females (PCGMK 1655b); Shal, 35°54.1'N, 49°48.0'E, 18 July 2013, leg. G.M. Kashani

& B. Eshaghi, four males and three females (PCGMK 1662); Zia-abad, 36°00.6'N, 49°27.8'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, one male and fifteen females (PCGMK 1667b); Takestan to Qazvin, Kahak village, 36°06.7'N, 49°45.0'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, five females (PCGMK 1668b); Khakali, 36°08.2'N, 50°10.7'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, two females (PCGMK 1686b); Koohin, 36°18.6'N, 49°48.8'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1692a); Abhar, Darasajin village, 36°01.1'N, 49°14.2'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, two males (PCGMK 1701b); Dowlat-abad to Abgarm, 35°55.5'N, 49°02.9'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1704b); Abgarm, 35°48.6'N, 49°08.0'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, two females (PCGMK 1705b); Takestan to Danesfahan, 35°52.5'N, 49°31.1'E, 12 September 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1713b); Qazvin to Nikouieh, Charandagh village, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, one male (PCGMK 1764).

Remarks. The presence of *P. major* in Iran was formerly reported by Kashani (2014a) but this is the first time it is recorded from the province of Qazvin. This species can be observed in high numbers especially in cultivated areas.

Distribution. From middle Europe to Central Asia; Iran.

Protracheoniscus ehsani Kashani, 2014

Material examined. Saveh to Boin-Zahra, Vardeh village, 35°15.2'N, 50°16.4'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, eight males and five females (PCGMK 1652); Boin-Zahra to Sagzabad, 35°48.1'N, 49°52.5'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, seven females (PCGMK 1658); Takestan to Shal, 35°54.1'N, 49°48.0'E, 18 July 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1663); Qazvin to Razmian, Barajin village, 19 July 2013, leg. G.M. Kashani & B. Eshaghi, eight females, two males and seven juveniles (PCGMK 1669); Qazvin to Razmian, Barajin village, 19 July 2013, leg. G.M. Kashani & B. Eshaghi, two females (IRIPP Iso.1048); 20 Km N Qazvin, 36°20.7'N, 50°10.7'E, 19 July 2013, leg. G.M. Kashani & B. Eshaghi, two males and seven females (PCGMK 1675); Khakali, 36°08.4'N, 50°10.7'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, eight males and thirteen females (PCGMK 1685); 28 Km to Kouhin, 36°16.9'N, 49°56.9'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1688); Nikouieh, 36°16.2'N, 49°31.7'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, two females (PCGMK 1695); Abhar to Darasajin village, 36°03.7'N, 49°13.4'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, three males and one female (PCGMK 1699); Darasajin village, 36°01.1'N, 49°14.3'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, three males (PCGMK 1700); Dowlat-abad, 35°58.6'N, 49°08.6'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, seven males and one female (PCGMK 1702); Dowlat-abad to Abgarm, Bouzandan village, 35°55.5'N, 49°02.9'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, two

males (PCGMK 1703); Abgarm to Takestan, Sagzenab village, 35°47.6'N, 49°22.8'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, six males and sixteen females (PCGMK 1710); Takestan to Danesfahan, 35°52.5'N, 49°31.1'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, two males and two females (PCGMK 1712); Nikouieh, Changooreh village, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, one female (PCGMK 1767); Qazvin to Nikouieh, Changoureh village, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, four males and six females (PCGMK 1769); 4 km to Nikouieh, 4 June 2014, leg. G.M. Kashani & B. Eshaghi, three males and eleven females (PCGMK 1771).

Remarks. This species was recently described from central parts of Iran (Kashani, 2014b). Here more sampling localities for the province of Qazvin are provided.

Distribution. Central Iran.

***Protracheoniscus sarii* sp. n.**

http://zoobank.org/334BDA58-C792-4808-8B47-F70AE0C55B75

Figures 1–2

Material examined. Holotype: male, 7 mm, Qazvin, Khakali, 36°08.2'N, 50°10.6'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, (ZUTC 5326).

Paratypes. Same data as holotype, one male (IRIPP Iso-1059); same data as holotype, one male (PCGMK 1684); Mali-Abad to Gheshlagh, 36°03.9'N, 50°19.7'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one female (IRIPP Iso-1055); Mali-Abad to Gheshlagh, 36°03.9'N, 50°19.7'E, 10 September 2013, leg. G.M. Kashani & B. Eshaghi, one male and two females (PCGMK 1682); 3 km to Avaj, 35°35.5'N, 49°13.3'E, 11 September 2013, leg. G.M. Kashani & B. Eshaghi, one male (PCGMK 1708).

Diagnosis. Head with short lateral and developed rounded median lobes. Male pleopod exopodite I with a truncate apex; endopodite I apex bent outward, equipped with some small setae.

Description. Maximum length of male and female 9 mm. Color brown with the usual pale muscle spots. Body outline as in Fig. 1A. Cephalon with rounded median lobe, protruding from the shorter lateral ones (Fig. 1B). Antenna long, surpassing the posterior margin of pereon tergite II; fifth article of peduncle as long as flagellum, with length: width ratio 6:1; flagellum with two articles, proximal article as long as the distal one (Fig. 1D). Pereopod I carpus with depression on rostral surface equipped with slender scales; propodus narrow and long, proximal part of sternal margin slightly concave with dense small scales, distal part bearing spine setae; pereopods I–VII dactylus with one dactylar and one ungual setae (Fig. 1E, F).

Pereon smooth. Pereon tergite I with rounded posterolateral margin. Noduli laterales on pereonites I to IV distinctly more distant from the lateral margins than those on pereonites V to VII (Fig. 1A).

Pleon narrower than pereon (Fig. 1A). Telson short, with distal part triangular bearing acute apex, slightly surpassing uropod protopodites (Fig. 1C). Uropod exo-

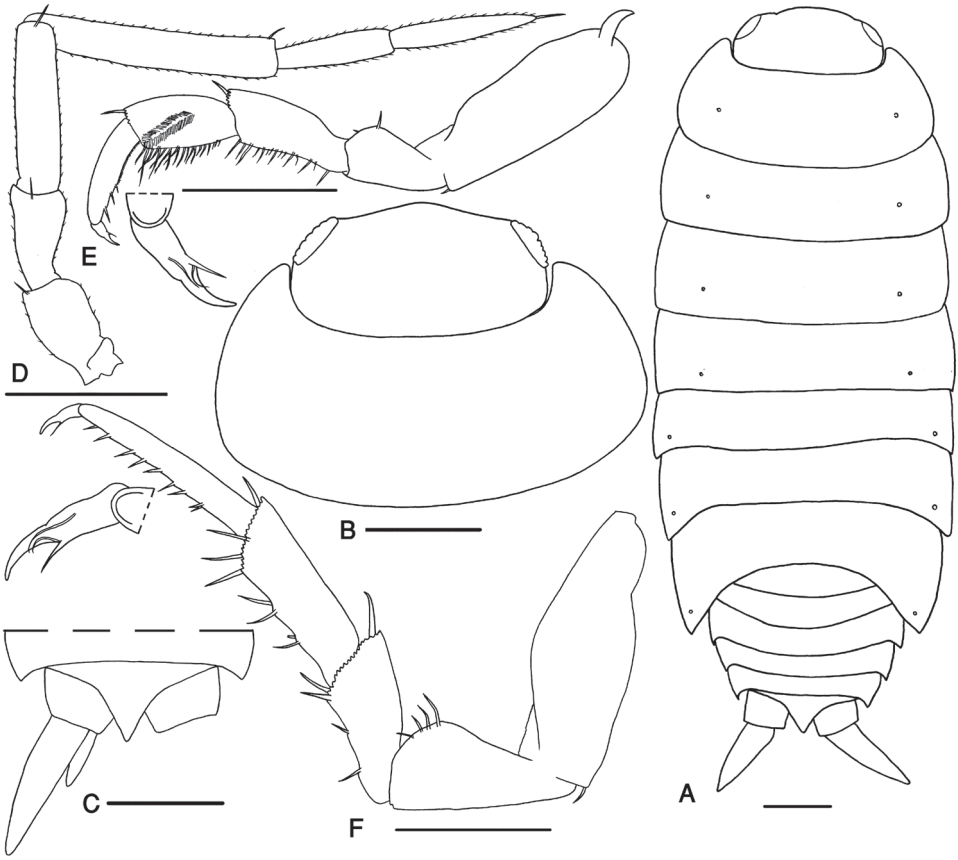

Figure 1. *Protracheoniscus sarii* sp. n., male, paratype. **A** body outline with position of noduli laterales **B** cephalon and first pereonite **C** telson and uropods **D** antenna **E** pereopod 1 **F** pereopod 7. Scale = 1 mm.

podites almost 1.5 times as long as telson (Fig. 1C). Pleopod exopodites I–V with monospiracular covered lungs (Fig. 1B–F).

Male: Pereopods I–III merus and carpus with brushes of setae (Fig. 1E). Pereopod VII ischium triangular, with straight ventral margin, merus and carpus equipped with strong spines on sternal and distal margins (Fig. 1F). Pleopod exopodite I with long hind lobe and truncate distal margin (Fig. 2B); endopodite I straight with apical part triangular, bent outwards and equipped with small setae (Fig. 2A). Pleopod endopodite II slightly longer than exopodite; exopodite triangular with a line of strong setae on outer margin (Fig. 2C). Pleopod exopodites III–V as in Fig. 2D–F.

Etymology. The name of the species is after Dr. Alireza Sari, professor in animal biosystematics, the University of Tehran, Iran.

Remarks. *Protracheoniscus sarii* sp. n. is superficially similar to *P. ehsani* but differs in lacking the ridge on the dorsal margin of pereopod VII carpus, and the shape of pleopod endopodite I. Ecologically, this species is associated with relatively humid microhabitats.

Distribution. Central Iran.

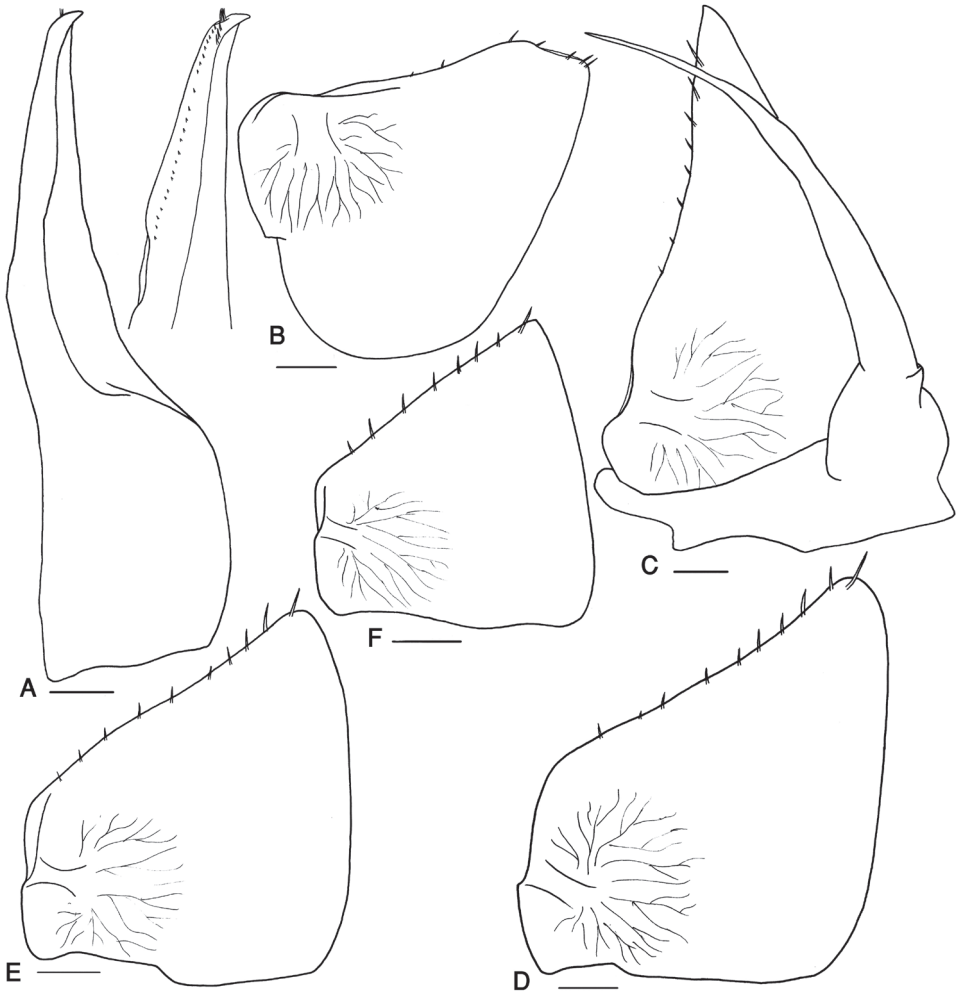

Figure 2. *Protracheoniscus sarii* sp. n., male, paratype. **A** pleopod endopodite I **B** pleopod exopodite I **C** pleopod II **D** pleopod exopodite III **E** pleopod exopodite IV **F** pleopod exopodite V. Scale = 0.1 mm

Discussion

Several papers have been published on terrestrial isopod fauna of Iran, however, most parts of the country have not been properly investigated and certainly more taxa are present. Prior to this study, 35 species belonging to 21 genera and 11 families were reported from Iran. According to the present knowledge, two genera, *Brevurus* Schmalfuss, 1986 and *Pseudorthometopon* Schmalfuss, 1986, and thirteen species, *Psachonethes elbursanus* Schmalfuss, 1986, *Trachelipus azerbaidzhanus* Schmalfuss, 1986, *T. pieperi* Schmalfuss, 1986, *Cylisticoides rotundifrons* (Schmalfuss, 1986), *Hemilepistus schirasi* Lincoln, 1970, *H. taftanicus* Kashani, Sari & Hosseinie, 2010, *Mongoloniscus persicus* Kashani, 2014, *Protracheoniscus gakalicus* Kashani, Malekhosseinie & Sadeghi, 2013,

P. ehsani Kashani, 2014, *Brevurus masandaranus* Schmalfuss, 1986, *Porcellio rubidus* Budde-Lund, 1885 (nomen dubium), *Schizidium persicum* Schmalfuss, 1986 and *Pseudorthometopon martensi* Schmalfuss, 1986, are endemic to Iran.

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RESEARCH ARTICLE

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Does temperature and oxygen affect duration of intramarsupial development and juvenile growth in the terrestrial isopod *Porcellio scaber* (Crustacea, Malacostraca)?

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Abstract

According to the temperature-size rule (TSR), ectotherms developing under cold conditions experience slower growth as juveniles but reach a larger size at maturity. Whether temperature alone causes this phenomenon is unknown, but oxygen limitation can play a role in the temperature-size relationship. Oxygen may become limited under warm conditions when the resulting higher metabolism creates a greater demand for oxygen, especially in larger individuals. We examined the independent effects of oxygen concentration (10% and 22% O_2) and temperature (15 °C and 22 °C) on duration of ontogenic development, which takes place within the maternal brood pouch (marsupium), and juvenile growth in the terrestrial isopod common rough woodlouse (*Porcellio scaber*). Individuals inside the marsupium undergo the change from the aqueous to the gaseous environment. Under hypoxia, woodlice hatched from the marsupium sooner, but their subsequent growth was not affected by the level of oxygen. Marsupial development and juvenile growth were almost three times slower at low temperature, and marsupial development was longer in larger females but only in the cold treatment. These results show that temperature and oxygen are important ecological factors affecting developmental time and that the strength of the effect likely depends on the availability of oxygen in the environment.

Keywords
Temperature-size rule, Oxygen, Ontogenic development, Crustacea, Oniscidea

Introduction

One of the most widespread patterns in biology is the temperature-size rule (TSR), which predicts slower growth and larger adult size for ectotherms growing in cold environments (Atkinson 1994). The TSR has been confirmed in more than four-fifths of studied species, including bacteria, protists, plants, invertebrates and ectothermic vertebrates (Atkinson 1994; Forster et al. 2012). Although a considerable number of the species investigated do not follow the TSR (Aguilar-Alberola and Mesquita-Joanes 2014; Kingsolver et al. 2007; Walczynska and Serra 2014) or show the reverse pattern (Diamond and Kingsolver 2010; Walters and Hassall 2006), research has been mainly directed toward finding a single mechanism that can explain the rule as well as its exceptions (Angilletta et al. 2004b; Cabanita and Atkinson 2006; Forster et al. 2012; Klok and Harrison 2013b). Since the formulation of the TSR, various adaptive and non-adaptive physiological mechanisms have been proposed (see Ray 1960 for his earlier finding). Von Bertalanffy (1960) and Perrin (1995) argued that the different temperature sensitivity of the physiological processes that affect energy uptake and utilisation may affect growth and produce the TSR (but see Angilletta and Dunham 2003). Adult body size is the product of growth rate and development time, so it has been suggested that individuals with higher temperature thresholds for development than growth should follow the TSR (van der Have and De Jong 1996; Walters and Hassall 2006; Zuo et al. 2012). Otherwise, differences in adult body size might be driven by other factors that correlate with ambient temperature, such as season length (Ejsmond et al. 2010), mortality (Angilletta et al. 2004b) or oxygen availability (Atkinson et al. 2006; Klok and Harrison 2013a). Environmental oxygen concentration has been shown to correlate with body size in aquatic amphipods, red swamp crayfish and rotifers (Bonvillain et al. 2015; Kielbasa et al. 2014; Peck and Chapelle 2003); individuals that were reared in hypoxic conditions experienced reduced growth rate and increased development time, resulting in a smaller final body size (Frazier et al. 2001; Harrison et al. 2006). The interplay between oxygen availability in the environment and the oxygen requirements of the organism, which are both dependent on thermal conditions, may explain the patterns that are consistent with the TSR (Atkinson et al. 2006; Hoefnagel and Verberk in press; Verberk et al. 2011; Walczynska et al. in press). Because the high metabolic oxygen demands of large individuals increase more rapidly at high temperatures (Verberk et al. 2011; Woods 1999), oxygen limitation is expected to be stronger under warm conditions (Chapelle and Peck 1999; Verberk and Atkinson 2013) and at later stages of ontogeny (Aguilar-Alberola and Mesquita-Joanes 2014). Consequently, these different size- and temperature-dependent oxygen requirements of ectotherms may favour a smaller body size in warm environments and a larger size in cold ones (Atkinson et al. 2006; Frazier et al. 2001; Walczynska et al. in press).

Growth rate of crustaceans is affected by the combination of internal and external factors (Hartnoll 2001). Considering the external factors, temperature and food supply are the most important drivers of variation in growth rate. Generally, lower temperature or food supply slows down growth, but the underlying proximate mechanisms are poorly understood in crustaceans (Hartnoll 2001). We used the terrestrial isopod species common rough woodlouse (*Porcellio scaber* Latreille, 1804) to examine the effect of ambient oxygen and temperature on duration of development within the maternal brood pouch (marsupium) and on juvenile growth. Our experimental approach enabled us to disentangle the independent effects of temperature and oxygen, which are otherwise correlated in nature. Terrestrial isopods use a two-stage gas exchange system in which oxygen initially dissolves in the haemolymph and is subsequently delivered to the tissues (Wright and Ting 2006), which may lead to oxygen limitation at higher temperatures (Klok et al. 2004). Furthermore, early ontogenetic development in isopods takes place in an aqueous environment inside the brood pouch (Surbida and Wright 2001) where oxygen pressure is much lower than that of the ambient air (Strathmann 1990). At later stage of intramarsupial development, individuals undergo the change from the aqueous to the gaseous environment by absorbing the marsupial fluid (Hoese and Janssen 1989). We expected that higher temperatures would decrease the development time within the marsupium and speed up juvenile growth, which is a general trend for ectotherms, but we wanted to test whether the level of oxygen moderates this thermal effect.

Materials and methods

Collection and maintenance of isopods

Common rough woodlice (*P. scaber*) were collected in the autumn of 2013 in Kraków, Poland. Adult males and females were kept in separate plastic boxes (205 × 150 × 97 mm) in a temperature-controlled room at 15 °C (12-h day) and 8 °C (12-h night). The bottoms of the boxes were covered with wet sand and pieces of a clay pot were provided as shelter, and the animals were supplied *ad libitum* with alder and ash leaves collected from a nearby forest. After two weeks in these conditions, males (n = 1120) and females (n = 1400) were combined and transferred to new boxes for copulation and egg-laying. Fifty females and forty males were placed in each box and distributed among the experimental conditions. The photoperiod was changed to 16 h L:8 h D to initiate reproduction (McQueen and Steel 1980).

Experimental conditions

Animals were reared in two climate chambers (15 °C and 22 °C, POL-EKO APARATURA, Sp.j., Poland), which contained two plexi-chambers (40 × 50 × 55 cm, YETI –

Agencja Reklamy, Poland) with either normoxic (22%) and hypoxic (10%) conditions. This experimental set-up gave us four temperate and oxygen combinations: 15 °C and 22% oxygen, 15 °C and 10% oxygen, 22 °C and 22% oxygen, and 22 °C and 10% oxygen. Oxygen levels were regulated (ROXY-4 four channel gas regulator, Sable Systems Europe GmbH, Germany) using oxygen (normoxic) or nitrogen gas (hypoxic), and the gases were provided by Air Products Sp. z o.o., Poland. Relative humidity was maintained at 75% by a separate dew point generator for each of the four environmental conditions (DG-4, Sable Systems Europe GmbH, Germany), and temperature and relative humidity settings were confirmed with Hygrochron iButtons (Maxim/Dallas Semiconductor, USA). The relative humidity inside the rearing boxes reached 98%, which was the humidity measured in the wild colony of isopods in Kraków.

Gravidity and parturition

Once per week, females were checked for the presence of a marsupium. Gravidity in *P. scaber* is characterised by the formation of a brood pouch on the ventral side of the body, and inside the marsupium, offspring undergo twenty discrete intramarsupial stages (Milatovič et al. 2010; Wolff 2009). After hatching from marsupium, offspring undergo two postmarsupial stages, postmarsupial mancae and juveniles (Tomescu and Craciun 1987). Each individual gravid female was transferred to a separate box (52 × 48 mm, 100 ml) containing wet sand, a piece of clay pot and alder and ash leaves as food, and the boxes were checked for the presence of newborns once per week. After releasing the mancae from marsupium, females were removed from the boxes and weighed alive to the nearest 0.01 mg (XP26, Mettler Toledo, Switzerland). The duration of intramarsupial development was defined as the time between the observation of a marsupium and the observation of offspring. Newly released mancae were maintained in the box without handling for a period of nine weeks, and only leaves were added as food if necessary. One sacrificed adult conspecific was added to each box two weeks after marsupium release to facilitate the acquisition of digestive tract symbionts, which are important to the early growth and survival of juvenile woodlice (our unpublished data). At the ages of nine and thirteen weeks after leaving the marsupium, a subsample of ten juveniles from each clutch (box) was weighed alive to the nearest 0.001 mg, and mean offspring mass was calculated by dividing the combined mass by the number of offspring.

Statistical analyses

Statistical analysis was performed with R software (R Core Team 2014), and the graphs were made using Statistica10 (StatSoft, Inc. 2011). Prior to analysis, normality and the homogeneity of variance were checked; based on the type of data female post-parturial mass and duration of marsupial development data were logarithmically and square root transformed, respectively.

The duration of marsupial development was analysed by ANCOVA with oxygen and temperature as fixed factors and the mass of the mother as numeric covariate, and all possible interactions. In total, 401 gravid females were used in the analyses (22 °C normoxia, n = 99; 22 °C hypoxia, n = 49; 15 °C normoxia, n = 153; 15 °C hypoxia n = 100). The best model was obtained following stepwise removal of all non-significant interactions (temperature × oxygen, oxygen × maternal mass and oxygen × temperature × maternal mass).

Juvenile growth was analysed with a generalised linear mixed model (GLMM); oxygen, temperature and time since leaving the marsupium were fixed factors, and box number was a random factor. Juvenile mass data were transformed with natural logarithms. In total, we analysed 369 clutches (22 °C normoxia, n = 145; 22 °C hypoxia, n = 53; 15 °C normoxia, n = 100; 15 °C hypoxia, n = 71). All non-significant interactions (temperature × oxygen, oxygen × time and oxygen × temperature × time) were removed in a stepwise manner from the model and were not included in the final analysis.

Results

Duration of intramarsupial development

Females reared in hypoxia released their offspring from their marsupia significantly sooner than females under normoxia (15 °C: 59.3 days normoxia, 56.8 days hypoxia; 22 °C: 23.1 days normoxia, 22.4 days hypoxia; p = 0.019; Table 1, Fig. 1). Generally, the duration of intramarsupial development in warm conditions was half that in the cold temperature (23 vs. 58 days; Fig. 1), but female post-parturial mass and temperature had an interactive effect on the duration of marsupial development (p < 0.001; Table 1, Fig. 2), which caused apparent lack of significant effect of temperature (Table 1). In cold conditions, marsupial development time increased with the mass of the mother but was independent in the warm environment.

Table 1. Effects of temperature and oxygen on the length of marsupial development (ANCOVA) and juvenile mass (GLMM) in the isopod *Porcellio scaber*. Female post-parturial mass and juvenile mass were logarithmically transformed, and the duration of intramarsupial development was square-root transformed.

Effect	**Df**	**F**	**p**
Duration of intramarsupial development			
Temperature	1	0.3	0.568
Oxygen	1	5.6	**0.019**
Female post-parturial mass	1	28.1	**< 0.0001**
Temperature × female post-parturial mass	1	13.7	**< 0.001**
Error	393		
Juvenile mass			
Temperature	1	1205.2	**< 0.0001**
Oxygen	1	3.5	0.064
Time	1	2700.8	**< 0.0001**
Temperature x time	1	174.9	**< 0.0001**

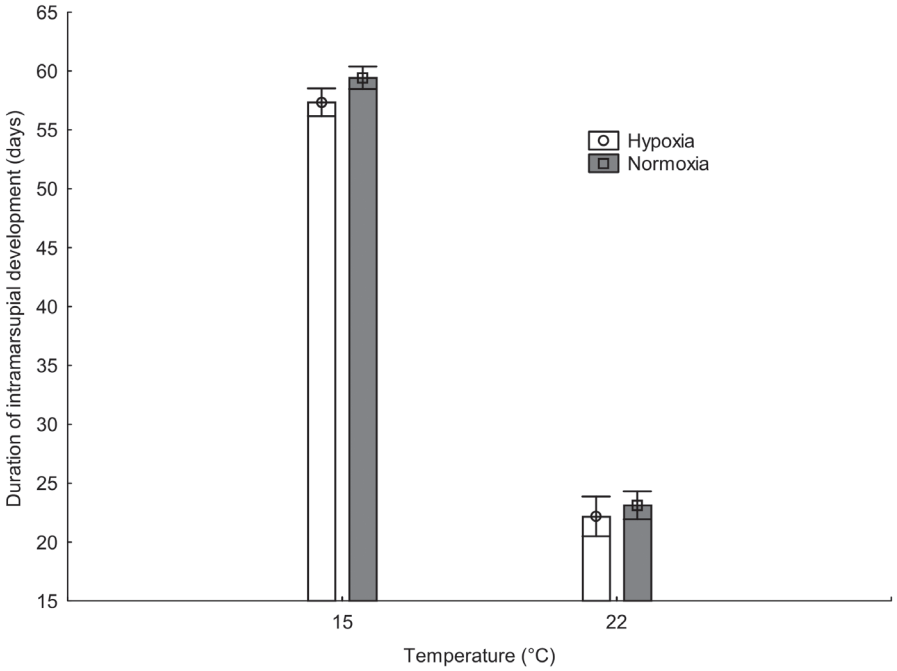

Figure 1. The effect of normoxia and hypoxia in cold and warm environment on the duration of intramarsupial development (expected marginal means ±CI) in the isopod *P. scaber*.

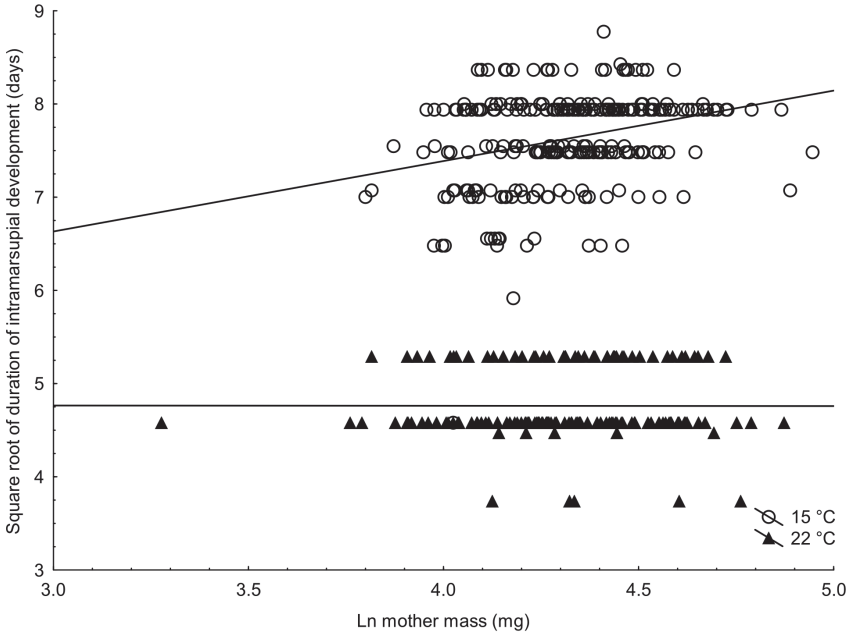

Figure 2. The relationship between female post-parturial mass and the duration of marsupial development in cold and warm environment in the isopod *P. scaber*.

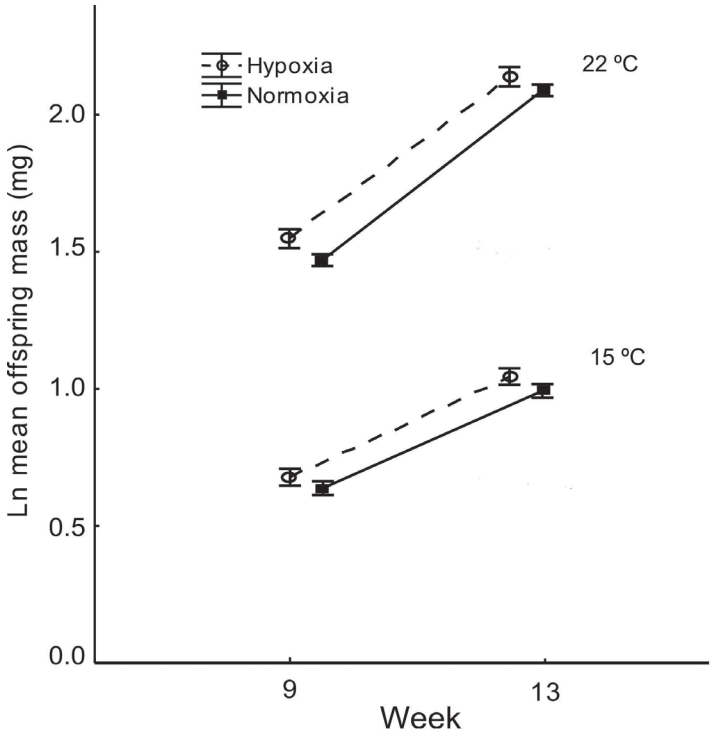

Figure 3. The effect of normoxia and hypoxia in cold and warm environment on juvenile growth (expected marginal means±CI) in the isopod *P. scaber*.

Juvenile growth

Juveniles grew faster in warm than in cold temperatures as indicated by the significant interaction between temperature and time ($p < 0.0001$; Table 1, Fig. 3). Oxygen concentration did not significantly affect growth ($p = 0.064$; Table 1, Fig. 3).

Discussion

We found that the duration of intramarsupial development of woodlice depended on temperature and oxygen level, with the latter effect being small, but statistically significant. Juvenile growth depended on temperature with a marginally significant effect of the level of oxygen ($p = 0.064$). Woodlice exposed to hypoxia completed their marsupial development sooner, and despite the shorter developmental time, juveniles under hypoxia were consistently slightly larger at both temperatures and time periods (after 9 and 13 weeks). However, because the difference was not significant, we can only conservatively state that hypoxia does not slow juvenile growth. Low temperature extended marsupial development and retarded juvenile growth; the two processes were almost two / three times slower at 15 °C than at 22 °C. Different behavioural, physiological

and biochemical mechanisms may explain these patterns. For example, individuals in low temperature may just have reduced their food intake (Rombke et al. 2011), and or in alternative, low temperature may affect through decreased metabolism (Iguchi and Ikeda 2005), for example by reducing the activity of digestive enzymes as found in the mud crub *Scylla serrata* (Pavasovic et al. 2004). These results showed that temperature accelerates both development and growth whereas hypoxia shortens development time regardless of temperature.

The shortened development time under warm conditions in *P. scaber* is consistent with the experimental evidence of faster development at high temperatures in a variety of crustacean species (Forster and Hirst 2012), but see Klok and Harrison (2013b), including shorter marsupial development of female Mysidacea (Crustacea) living in warm regions (Wittmann 1984). However, whether oxygen mediated this temperature response was not examined in these studies, but in accordance with the oxygen-driven TSR, we would expect smaller hatchlings under hypoxia and larger hatchlings under normoxia. Because individuals hatched earlier under hypoxic conditions, the similar body mass after nine weeks of growth in both oxygen treatments can be explained by either similar masses at hatching, which was not studied because the hatchlings were too delicate to weigh, or compensatory growth in juveniles reared in hypoxia, as observed in shrimp (*Fenneropenaeus chinensis*) (Wei et al. 2008). Because constraints on growth should arise later in ontogeny when animals are bigger and oxygen limitations are stronger (Hoefnagel and Verberk in press; Richmond et al. 2006), one could expect to find differences in growth rate at the later stages of ontogenetic development (Aguilar-Alberola and Mesquita-Joanes 2014; Forster et al. 2012). We cannot exclude that further growth until maturation would reveal such a hypothesised oxygen limitation. Therefore, applied hypoxia (10% O_2) might be sufficient to set oxygen limits on the rate of development but not during the early growth of *P. scaber* (see also Klok et al. 2004; Stevens et al. 2010).

The observed effects of differential oxygen between the rate of marsupial development on one hand and juvenile growth on the other may be related to dissimilar oxygen availability in the aqueous and gaseous environments. The early development of isopods, as well as those of other crustacean groups (e.g., Amphipoda and Mysidacea), occurs in a fluid-filled brood pouch, which protects the early stages of development against desiccation, osmotic stress and mechanical damage (Ouyang and Wright 2005; Surbida and Wright 2001). Special maternal extensions into the marsupium, called cotyledons, have been suggested to supply offspring with oxygen and nutrients (Hoese and Janssen 1989). As oxygen uptake is far more challenging in water than in air due to its higher viscosity and density (Strathmann 1990), oxygen limitations are expected to be stronger in aquatic environments (Hoefnagel and Verberk in press; Verberk et al. 2011; Walczynska et al. in press). Indeed, Forster et al. (2012) found stronger support for the TSR in aquatic than in terrestrial environments (but see Klok and Harrison 2013 for evidence of equal support). Oxygen limitations due to constraints on oxygen diffusion have mainly been found in species that carry brood pouches with tightly packed embryos (Baeza and Fernandez 2002; Fernandez et al. 2002; Lee and

Strathmann 1998). If lower oxygen diffusion inside a brood pouch increases the risk of mortality, juveniles may hatch from the marsupium sooner, an effect we observe under hypoxia in both temperatures. However, we are unable to differentiate whether shorter duration of intramarsupial development in hypoxia is caused by faster developmental rate of mancae or individuals perceived hypoxia level as a stress signal and they simply left marsupium sooner. Besides the unknown cause, our results provide support that oxygen is a limiting factor in the early stages of ontogenetic development in *P. scaber* that occur in the liquid phase.

The duration of intramarsupial development was not only affected by temperature and oxygen, but also by female mass; in the cold temperature, larger females incubated their progeny longer. Longer marsupial development in larger females agrees with findings for other species of terrestrial isopods: *Armadillidium vulgare*, *Cylisticus convexus* and *P. scaber* (Hatchett 1947). In contrast, a negative correlation between female mass and incubation period was found in *Porcellio laevis* (Lardies et al. 2004). Because embryonic development takes place in a maternal brood pouch, its length might not only be affected by environmental factors but also by the female (i.e., a maternal effect Mousseau and Fox 1998). Females can adopt different strategies in cold and warm environments to increase the fitness of their offspring and their future prospects for reproduction (Angilletta et al. 2004a). A shorter activity window in cold environments may limit the reproductive opportunities for females (Adolph and Porter 1996), so smaller females that produce relatively smaller clutches may increase their reproductive activity by accelerating embryonic development and producing additional clutches (for different isopod species see Warburg 2013). Untouched by the effect of female mass on subsequent juvenile growth and its possible explanation, this study demonstrates that maternal factors must be considered to be of general importance when determining if animals follow the TSR.

Conclusion

Our data show that oxygen level affects duration of intramarsupial development of the terrestrial isopod *P. scaber* in an unexpected way; development is shorter under lower levels of oxygen. Although we cannot exclude the possibility that mancae hatched sooner at earlier developmental stage compared to mancae in normoxia, our results suggest that oxygen availability is crucial for development in marsupium, and future studies may be directed towards determining the developmental stages of freshly hatched mancae reared in different experimental conditions. Our results further suggest that oxygen level rather does not affect growth rate after hatching. The size of the mother may affect the rate of embryonic development to some extent, but that effect depends on the thermal environment. Duration of intramarsupial development and early growth rate are accelerated in warm compared to cold environment. We might expect that such a strong effect on early life stages may have important consequences for subsequent life stages. To what extent our observed patterns may explain life-histo-

ry strategies employed by terrestrial isopods living in different thermal environments and how this in turn may affect their range expansion and geographical distribution may provide interesting approach for future investigations.

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Histological studies on the marsupium of two terrestrial isopods (Crustacea, Isopoda, Oniscidea)

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Abstract

The marsupium, a brood pouch in peracarid crustaceans (Crustacea, Malacostraca) has evolved in terrestrial environment for providing nutrition and optimal conditions for embryogenesis. In the present study we give details on the histology and ultrastructure of its constituting elements such as oostegites and cotyledons. Marsupia of two different eco-morphological types of woodlice, namely the non-conglobating species *Trachelipus rathkii* Brandt, 1833 and the conglobating species *Cylisticus convexus* De Geer, 1778 were investigated. Light microscopic (LM) studies showed some differences in the main structure of the two species' brood pouch: in *T. rathkii*, a 'clinger' type woodlice, the oostegites bend outwards during brood incubation as growing offspring require more space, while in *C. convexus*, a 'roller' type isopod, the sternites arch into the body cavity to ensure space for developing offspring and still allowing conglobation of the gravid females. The quantitative analysis of the oostegites' cuticle proved that the outer part is about 2.5 - 3 times thicker compared to the inner part in both species. Electron microscopic (TEM) examinations show only small histological differences in the oostegites and cotyledon structure of the two species. Cellular elements and moderately electron dense fleecy precipitate are found in the hemolymph space between the two cuticles of oostegites. The cells contain PAS positive polysaccharide areas. TEM studies revealed some differences in the cotyledon ultrastructure of the two species. Cotyledons of *T. rathkii* consist of cells with cristate mitochondria and granular endoplasmic reticulum with cisterns. Cotyledons of *C. convexus* consist of cells with densely cristate mitochondria and ribosomes attached to vesicular membrane structures. In both species cells with electron dense bodies were observed. We conclude that - besides the differences in marsupial shapes - the fine structure of the oostegites and cotyledons is hardly affected by the eco-morphological type, specifically the conglobating or non-conglobating character of the studied species.

Keywords
Oniscidea, oostegite, cotyledon, light microscopy, electron microscopy, eco-morphological type

Introduction

During evolutionary land adaptation Oniscidea have developed various morphological, physiological and behavioral solutions to cope with the challenges of terrestrial life (e.g. desiccation, respiration and reproduction), such as pleopodal lungs, water-resistant cuticle and water conducting system. Concerning reproduction they show an extended parental care (XPC), which is a widespread phenomenon in crustaceans. In the majority of peracarid taxa with XPC, offspring are carried in the female's body, developing from egg to manca larval stage in a temporal brood pouch (marsupium) (Thiel 2003). Care for late developmental stages appears to be an important adaptation in terrestrial environment.

The brood pouch originally evolved for mechanical protection of eggs and developing embryos under water conditions (Steele 1991). In terrestrial environment the ovigerous females produce a microenvironment in the marsupium, providing fluid and oxygen for the developing young (Hoese 1984, Hornung 2011, Linsenmair 1989, Warburg 1987, Warburg and Rosenberg 1996). The brood pouch is formed during the parturial molt (Suzuki and Yamasaki 1989, Suzuki 2002). Hoese (1984) described two types of the Oniscidean marsupium: the amphibian type and the terrestrial one. In the more primitive amphibian type, the marsupium is open both anteriorly and posteriorly, similarly to the aquatic type, and it is connected to a water-conducting system. Fluid circulates in the water-conducting system, also passing through the marsupium. In the terrestrial type, the brood pouch is not connected to the water-conducting system; however the marsupial cavity is filled up with fluid.

Five pairs of oostegites cover the marsupium, which is tightly sealed ventrally and laterally. Oostegites are leaf-like, overlapping appendages, basally fused with the pereomeres (Hoese 1984, Hoese and Janssen 1989, Suzuki and Yamakasi 1991). Suzuki and Yamakasi (1991) concluded that oostegite formation is controlled by ovarian processes. The factor that stimulates oostegite formation may be the same that regulates vitellogenin synthesis.

The inner structure of the marsupium also differs among woodlice, depending on the phylogenetic position of the species. In some - more developed - species it is divided by segmental cotyledons, which are responsible for nutrition and oxygenation of the offspring (Akahira 1956, Hoese and Janssen 1989, Hornung 2011). Cotyledons are metameric outgrowths on thoracic segments 1-5, which develop only during the marsupial period from transverse ridges of the ventral epidermis. Their shapes and dimensions vary in different species and with the stage of the marsupial period (Hoese and Janssen 1989). Vandel (1925, 1942) recognized that Ligiidae, Trichoniscidae, and Tylidae never possess cotyledons, whereas Oniscidae, Porcellionidae, and Armadillidiidae always do. Lewis (1991) hypothesized that the number of cotyledons is related

to both phylogenetic position and habitat characteristics (e.g. drought). She found cotyledon numbers ranging from 4 to 28 per female, investigating several species.

Warburg and Rosenberg (1996) reported on a special structure in the conglobating Mediterranean species, *Armadillo officinalis* and *Schizidium tiberianum*. They found sacs inside the marsupial cavity connected to the marsupial roof. These sacs contained the developing eggs, embryos and mancas organized in small groups.

In species belonging to the 'roller' eco-morphological type (Schmalfuss 1984) the oostegites bend only slightly which allows ovigerous females still to conglobate. According to the stereo-microscopic studies of Appel et al. (2011) in such cases sternites arch into the body cavity to provide more space for the developing embryos. They studied the conglobating *Armadillidium nasatum*, *A. vulgare*, *Pudeoniscus birabeni*, *Circoniscus gaigei*, *Cubaris murina* and the non-conglobating *Neotroponiscus daguerri* and *N. carolii*. Conglobation leads to a displacement and compression of the female's internal organs, which may cause females to cease feeding in advanced gravidity stages. In non-conglobating ('clinger', 'runner') species the oostegites bend outwards during brood incubation.

The objective of the present paper was to compare the brood pouches of two basically different eco-morphological types (Schmalfuss 1984), by light- and electron microscopical techniques (LM, TEM).

Materials and methods

Examined species

The two investigated species were the non-conglobating 'clinger' type *Trachelipus rathkii* Brandt, 1833 and the conglobating 'roller' type *Cylisticus convexus* De Geer, 1778. According to Schmidt (2008) both species belong to the group of the Crinocheta, which is one of the five principal lineages of the Oniscidea. While *T. rathkii* is a member of the "Trachelipodidae", *C. convexus* belongs to the "Cylisticidae" group.

The ovigerous females of the examined species were hand collected in a deciduous forest (*Querco petraeae – Carpinetum*) of the Buda-mountains, near Budapest, Hungary, during their reproductive period (from May to June) in 2014.

Light microscopy

For light microscopic investigations (LM) two nearly same sized ovigerous females per species, in the identical marsupial stage, were fixed in an aqueous solution containing 4 % paraformaldehyde, 2 % glutaraldehyde and 0.1 M phosphate buffer (PB) for 48 hours, followed by rinsing in PB. After fixation, tissues were postfixed in 2% osmium tetroxide in 0.1 M PB for 6 hours. The samples were dehydrated through a graded series of ethanol (50% – 30 min, 70% – 3 h, 90% – 1 h, 100% – 1 day). After

dehydration the samples were kept in propylene oxide for 1 day, followed by infiltration in propylene oxide : Durcupan resin (1:1) overnight. Samples were infiltrated with Durcupan for 24 hours and embedded afterwards. Histological sections (1 μm) were cut with a Reichert ultramicrotome and stained with toluidine blue. Several samples from the oostegite were stained with periodic acid-Schiff reagent (PAS) to detect polysaccharides such as glycogen in tissues (2 specimen/species, 10 samples/specimen). The sections were photographed with a Leica microscope.

Transmission electron microscopy

For transmission electron microscopic (TEM) studies two ovigerous females (same size, identical stage) from both species were injected under the tergite with 12.5% glutaraldehyde (in 0.1 M cacodylate buffer). Dissected oostegites and cotyledons with some eggs were fixed in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (2 h) and postfixed in 1 % osmium tetroxide and 0.8% potassium ferricyanide. The samples were dehydrated in a graded series of ethanol (30% – 1 h, 50% – 1 h, 70% – 3 h, 90% – 1 h, 100% – 1 day). They were pre-embedded in the mixture of EPON and 100% aceton (1:1). Finally the pieces were embedded in 100 % EPON for 24 hours. Ultrathin sections (60 nm) were cut with a Reichert Ultracut ultramicrotome, studied and photographed with a JEOL 100 C electron microscope.

Data analysis and statistical methods

To compare and quantify thickness of the oostegites' outer and inner cuticles, 60 measurements (2 specimen, 3 sections, 10 measurements/section) of each investigated species were taken using the TEM micrographs (ImageJ and MS Excel software) (Csonka et al. 2013). In order to assess the relevancy of the difference in the oostegite outer and inner cuticle thicknesses we performed a one-way ANOVA test followed by a post-hoc Tukey-test on the cross section specific cuticle thickness (R 2.11.1 software).

The schematic drawings about the structural elements were made with the Inkscape vector graphics editor software.

Results

Structure of marsupium

The LM cross sections show several similarities but also some differences between the compared marsupial structures of the two eco-morphological types. In the 'clinger' *T. rathkii* the oostegites bend outwards (Fig. 1A, B). In the 'roller' *C. convexus* the ster-

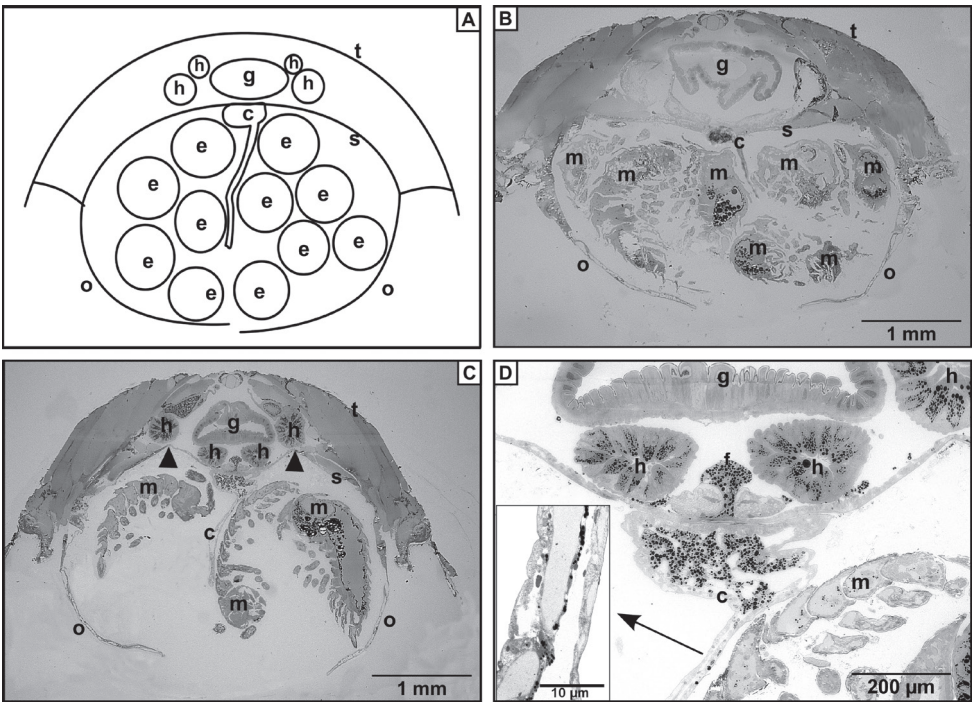

Figure 1. Cross sections of marsupium. **A** Schematic drawing of the brood pouch **B** Marsupium with developing mancas in the non-conglobating *Trachelipus rathkii* **C** Marsupium of conglobating *Cylisticus convexus* in the same stage. Note arching sternites (arrowheads) **D** Higher magnification image of the proximal part of the cotyledon in *C. convexus*. The cells are filled with darkly stained lipid droplets. Insert: Higher magnification reveals that along the longitudinal axis of cotyledon a beadlike array of lipid droplets lines up. Legends: c – cotyledon, e – egg, f – maternal fat body, g – gut, h – hepatopancreas, m – manca, o – oostegite, s – sternite, t – tergite.

nites arch into the body cavity (Fig. 1C arrow heads). In the cross section of marsupial cavity the developing mancas and cotyledons are clearly recognizable (Fig. 1B, C). Both studied species have one cotyledon situated centrally on each of segments 2-5.

Structure of oostegite

Both species have five pairs of oostegites (on thoracic segments 1-5), that have the same structure. TEM micrographs show that the outer cuticle of the oostegites is 2.5–3 times thicker compared to the inner one in both species (Fig. 2). We confirmed the morphological observations with quantitative analysis (Table 1). The ANOVA test revealed that the values of cuticle thickness differ significantly when comparing the inner and outer cuticle of the oostegite ($p < 0.001$).

In both species the space between the inner and outer cuticle consists of cellular elements and hemolymph space (Fig. 2A, B). This inner structure is similar all along the

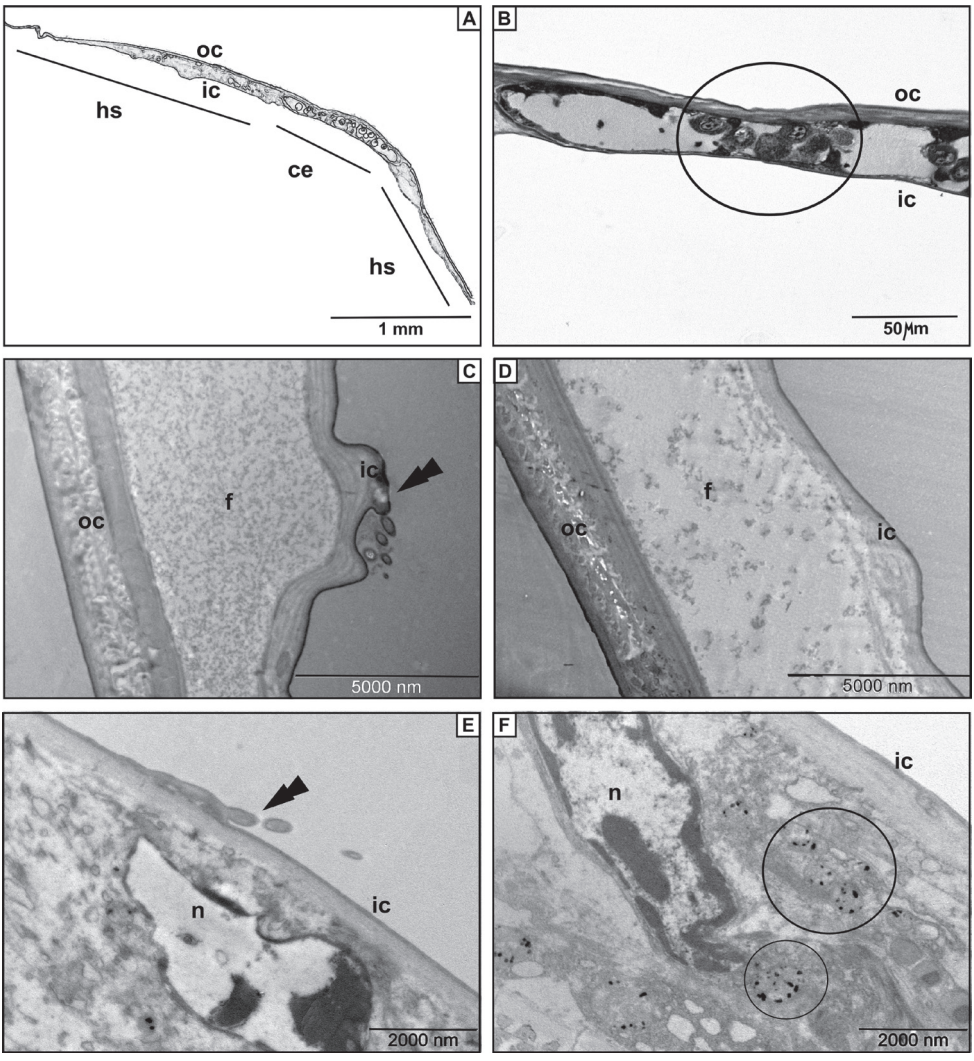

Figure 2. The structure of the oostegite. **A** Schematic drawing of the cross -sectioned oostegite **B** Semithin section after PAS staining with positive cytoplasm in the cells of the oostegite **C** An electron micrograph of the break between cells of *Trachelipus rathkii* oostegite. Note scale-like protrusion of the inner cuticle (arrow) **D** Identical detail in *Cylisticus convexus*. No protrusion was found **E** Cell in the oostegite of *T. rathkii* below a scale-like protrusion of the inner cuticle (arrow) **F** Cell in the oostegite of *C. convexus*. Note the membrane-bound electron dense inclusions. Legends: ce – cellular elements, f – fleecy precipitate, hs – hemolymph space, ic – inner cuticle, n – nucleus, oc – outer cuticle.

oostegite. In *T. rathkii* the hemolymph space contains moderately electron dense fleecy precipitate which is much less pronounced in *C. convexus* (Fig. 2C, D). The periodic acid-Schiff staining showed PAS positive cytoplasm in the cells within the hemolymph space of oostegite (Fig. 2B). Small scale-like protrusions are recognizable on several

Table 1. The mean thickness (nm) with standard deviation (SD) of the oostegites' outer (oc) and inner cuticle (ic) layer.

	Mean (oc)	SD (oc)	Mean (ic)	SD (ic)
T. rathkii 1	1828,3	± 233.6	724,76	± 241.3
T. rathkii 2	2001,6	± 183.2	697,5	± 212.3
C. convexus 1	2092,2	± 178.9	671,5	± 102.3
C. convexus 2	1997,3	± 189.6	699,4	± 199.1

electron micrographs of the oostegites' inner cuticle in *T. rathkii* (Fig. 2C, E). Similar structures were not observed in the oostegite of *C. convexus*. In the latter species, membrane-bound electron dense inclusions can be observed in the cells (Fig. 2F).

Structure of cotyledon

Cotyledons appear in the marsupium among developing offspring in both species. The maternal fat body and the cells of hepatopancreas contain densely stained lipid inclusions, similarly to the proximal part of the cotyledon, whereas along its longitudinal axis these line up in a bead-like array (Fig. 1D).

The electron micrographs (TEM) show cotyledons covered by an extremely thin cuticle (Fig. 3B). In both species these sternite outgrowths are built up by cells, which contain abundant mitochondria and rough endoplasmic reticulum (Fig. 3). We found obvious differences in the two investigated species concerning the structure of the mitochondria and the endoplasmic reticulum. The cotyledons in *T. rathkii* consist of cells with common cristate mitochondria and rough or granular endoplasmic reticulum with cisterns (Figs 3A, C). On the contrary in *C. convexus* there were cells with densely cristate mitochondria, their endoplasmic reticulum was dominated by rounded vesicle-like membranes instead of the common cisterns (Figs 3B, D).

We found cells in the cotyledon of both species with cytoplasm mainly characterized by the presence of several electron dense vesicles (Figs 3E, F). At the base of the cotyledon bundles of striated muscle fibers are present in both species (Fig. 3H).

Discussion

We examined the structure of the marsupium in two different eco-morphological types of woodlice: non-conglobating (*T. rathkii*) and conglobating (*C. convexus*). We predicted that differences between the two eco-morphological types are reflected in idiosyncratic morphological features of their brood pouches.

Light microscopic results here concurred with the statements of Appel et al. (2011). They compared the relationship between the morphology of the marsupium and the eco-morphological type with stereo microscopic techniques. By their findings

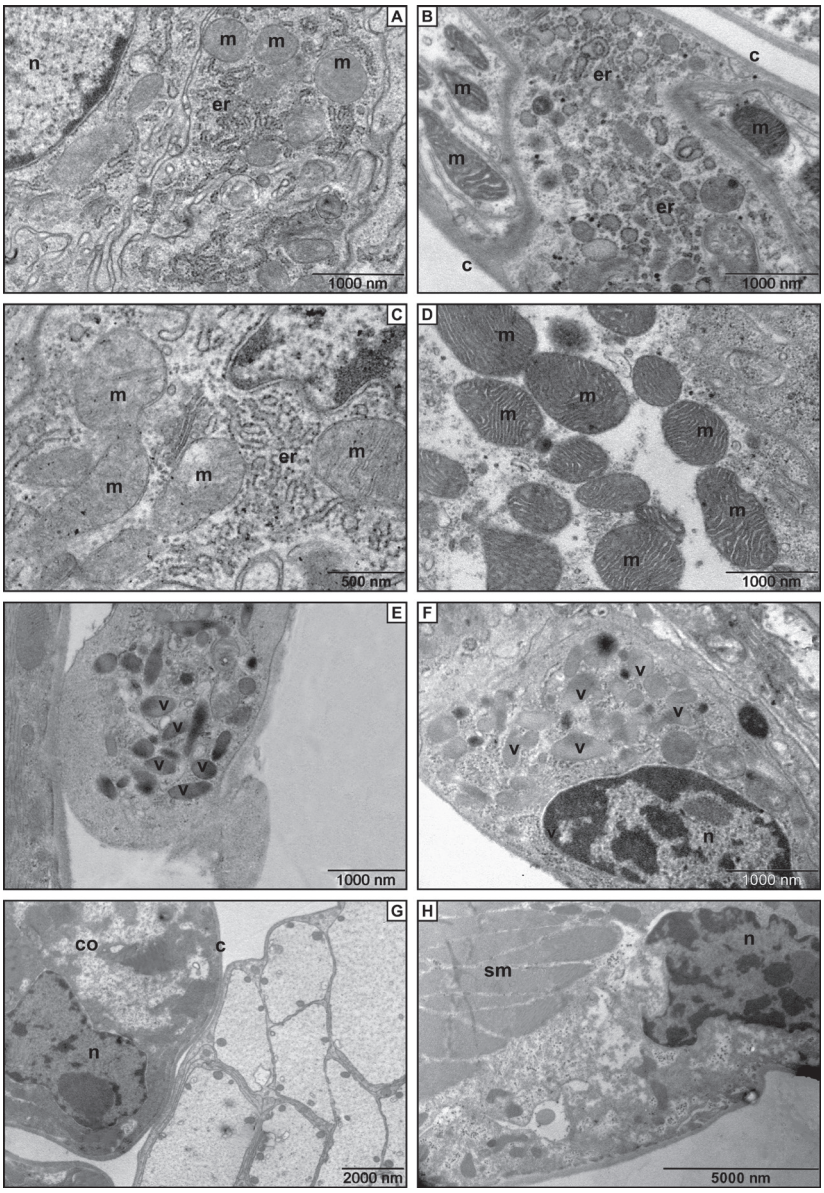

Figure 3. Electron micrographs of the cotyledon. **A** Fine structure of a cell from the medial portion of the cotyledon in *Trachelipus rathkii*. The most abundant cell organelles are mitochondria and rough endoplasmic reticulum **B** In the medial portion of the cotyledon the cells contain vesiculated rough endoplasmic reticulum and mitochondria (*C. convexus*) **C** Higher magnified detail of the cotyledon in *T. rathkii*. **D** High power micrograph of the cotyledon in *Cylisticus convexus*. Note the densely cristate mitochondria **E** Rounded ending of the cotyledon with electron dense vesicles (*T. rathkii*) **F** A cell with large vesicles containing moderately electron dense material (*C. convexus*) **G** Cotyledon ending of *T. rathkii* covered by a thin cuticle **H** Bundles of striated muscle fibers located at the base of cotyledon (*C. convexus*). Legends: c – cuticle, co – cotyledon, er – rough endoplasmic reticulum, m – mitochondria, n – nucleus, sm – striated muscle, v – vesicle.

the gravid females of conglobating species have sternites arching into the body cavity to provide more space for developing offspring. Warburg and Rosenberg (1996) recognized that in conglobating *A. officinalis* and *S. tiberianum* developing eggs, embryos and mancas are grouped into marsupial sacs. We did not find sac-like marsupial structure in the roller *C. convexus*, so that is not related to the conglobating form.

Treviranus and Treviranus (1816) were the first to describe cotyledons and oostegites of terrestrial isopods as parts of the marsupium. They assumed that cotyledons would be necessary for the nutrition of developing young. Patanè (1940, 1951) studied marsupium in *Porcellio laevis*, *Armadillidium cinereum*, *Trichoniscus pusillus provisorius*, *Ligia italica*, *Anilocra physodes* and assumed that marsupial fluid enters the brood pouch via cotyledons and oostegites and that the respiratory function predominates over that of nutrition. He mentioned that the ventral integument of the females is very thin and permeable. This could facilitate exchange of nutrients between body cavity and the marsupium. The outer cuticle of the oostegites has to be thick and impermeable to give protection against desiccation (Hoese 1984). Our quantitative analysis showed that in the two species studied here the outer cuticle is about 2.5–3 times thicker compared to the inner one in both species. In *Porcellio dilatatus* Luca (1965) discovered large secretory cells in the oostegites. According to our observations oostegites consist of cellular elements and hemolymph space. In the hemolymph space we observed moderately electron dense fleecy precipitate in varying amount in both species. This may be the solid part of the hemolymph precipitated during fixation. The PAS positive reaction of the oostegite cell cytoplasm may prove the presence of polysaccharides for the cuticle. Moreover, electron microscopic studies revealed small protrusions on the oostegites' inner cuticle in *T. rathkii*. These outgrowths could be engaged with sensory function monitoring the marsupial fluid content, although we failed to detect dendritic processes of neurons in contact with them. In this way they probably represent simply architectural elements like plaques or scales.

Akahira (1956) studied the epithelium and found a mucous mass inside and outside the cotyledons and some blood cells in the marsupium of *Porcellio scaber*. He suggested that cotyledons are storage organs for a mucous mass on which the embryos feed during development. Hoese and Janssen (1989) examined the brood pouch in isopods *Armadillo ausseli*, *Armadillidium vulgare*, *Hemilepistus aphganicus*, *H. reaumuri*, *Hyloniscus riparius*, *Philoscia muscorum*, and *Porcellio scaber*. These authors looked for indications of transport mechanisms through the cotyledon epithelium. They found that the histological features of cotyledon integument are identical with those of a locally differentiated and periodically active transport epithelium and it contains a portion of the maternal fat body. Our micrographs show also the maternal fat body, which is a fat-storing adipose tissue enclosing the ventral nerve cord (Hoese and Janssen 1989). Picaud and Souty (1980) and Picaud et al. (1989) detected vitellogenin synthesis in the fat body of *Porcellio dilatatus*. We observed densely stained lipid inclusions at the proximal part of the cotyledon. The structures are present also in the cells of hepatopancreas and the mancas, and resemble lipid droplets (Štrus and Blejec 2001). Our examination showed that cotyledons in both species are built up by cells, which contain

different types of mitochondria. The morphology of those is related to the functional states of cells. Mitochondria with tubes and vesicles are characteristic features of steroid producing cells in many species from ciliates to mammals, such as adrenal cortex and Leydig-cells (Secchi and Lecaque 1981, Bloom and Fawcett 1994). It may be that the cotyledon cells studied here are also engaged in the synthesis of similar molecules, although vesicular profiles could not be detected.

It is noteworthy that striated muscle fibers are present at the base of cotyledons. We suppose that they play an important role in the fixation of the cotyledons' basal part and they allow a certain degree of mobility.

We found only small histological differences in the oostegite and cotyledon structures of the two species with different eco-morphological background. Since both species belong to the same lineage of Oniscidea, these differences probably reflect to the physiological state of the animal, rather than the eco-morphological type. Further investigations are needed to compare several other species with different phylogenetic position in the future to make general statements.

Conclusions

Our findings show that the gross anatomy of the brood pouch in the examined species' agrees with that of species studied earlier. The main structure of the oostegites is similar in both species. Small protrusions of the oostegites' inner cuticle are recognizable only in *T. rathkii*. In the case of cotyledon the electron micrographs show differences in the two investigated species concerning the structure of the mitochondria and the endoplasmic reticulum, but these features can be related to their physiological state. The proximal part of the cotyledon contains dark vacuoles. These are lipid inclusions which might represent an energy storage site.

Acknowledgements

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RESEARCH ARTICLE

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Formation of the hindgut cuticular lining during embryonic development of *Porcellio scaber* (Crustacea, Isopoda)

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Abstract

The hindgut and foregut in terrestrial isopod crustaceans are ectodermal parts of the digestive system and are lined by cuticle, an apical extracellular matrix secreted by epithelial cells. Morphogenesis of the digestive system was reported in previous studies, but differentiation of the gut cuticle was not followed in detail. This study is focused on ultrastructural analyses of hindgut apical matrices and cuticle in selected intramarsupial developmental stages of the terrestrial isopod *Porcellio scaber* in comparison to adult animals to obtain data on the hindgut cuticular lining differentiation. Our results show that in late embryos of stages 16 and 18 the apical matrix in the hindgut consists of loose material overlaid by a thin intensely ruffled electron dense lamina facing the lumen. The ultrastructural resemblance to the embryonic epidermal matrices described in several arthropods suggests a common principle in chitinous matrix differentiation. The hindgut matrix in the prehatching embryo of stage 19 shows characteristics of the hindgut cuticle, specifically alignment to the apical epithelial surface and a prominent electron dense layer of epicuticle. In the preceding embryonic stage – stage 18 – an electron dense lamina, closely apposed to the apical cell membrane, is evident and is considered as the first epicuticle formation. In marsupial mancae the advanced features of the hindgut cuticle and epithelium are evident: a more prominent epicuticular layer, formation of cuticular spines and an extensive apical labyrinth. In comparison to the hindgut cuticle of adults, the hindgut cuticle of marsupial manca and in particular the electron dense epicuticular layer are much thinner and the difference between cuticle architecture in the anterior chamber and in the papillate region is not yet distinguishable. Differences from the hindgut cuticle in adults imply not fully developed

structure and function of the hindgut cuticle in marsupial manca, possibly related also to different environments, as mancae develop in marsupial fluid. Bacteria, evenly distributed within the homogenous electron dense material in the hindgut lumen, were observed only in one specimen of early marsupial manca. The morphological features of gut cuticle renewal are evident in the late marsupial mancae, and are similar to those observed in the exoskeleton.

Keywords
Development, digestive system, cuticle, extracellular matrix, embryo

Introduction

Epidermal body surfaces and ectodermal parts of the digestive system in crustaceans are covered by cuticle. The exoskeletal cuticle and the digestive tract cuticle are both chitin-based apical matrices, but differ in ultrastructural organization and functions. Exoskeletal cuticle in terrestrial isopod crustaceans is organized in three principal horizontal regions: epicuticle, exocuticle and endocuticle, that differ in the ultrastructural architecture and composition (Price and Holdich 1980, Compere 1990, Štrus and Compere 1996, Ziegler 1997, Štrus and Blejec 2001, Hild et al. 2008, 2009, Vittori and Štrus 2014). Epicuticle is the outermost thin layer, which is not mineralized and is composed predominantly of lipoproteins. The detailed ultrastructure reveals that epicuticle consists of an outer epicuticle, composed of several thin sublayers, and an inner epicuticle. Exo- and endocuticle are mineralized layers and display ''lamellae'', a pattern appearing due to helicoidal arrangement of chitin-protein fibers. The mineral component of tergite cuticle consists of calcite and magnesium-calcite, limited to the exocuticle, and amorphous calcium carbonate and amorphous calcium phosphate in the endocuticle (Hild et al. 2008, 2009, Neues et al. 2011, Seidl et al. 2011, Luquet 2012). Formation of the new exoskeletal cuticle has been extensively studied in molting adult crustaceans, including isopods (Price and Holdich 1980, Compere 1990, Štrus and Compere 1996, Ziegler 1997, Štrus and Blejec 2001, Vittori et al. 2012). Exoskeletal cuticle during early ontogenetic development of crustaceans was examined previously by *in vivo* or histological observations in decapods, branchiopods and amphipods (Freeman and Costlow 1980, Anger 1983, Snyder and Chang 1986, Belk 1987, Helluy and Beltz 1991). Several early ultrastructural studies, probing embryonic surface matrices, were made in branchiopods, isopods and decapods (Morris and Afzelius 1967, Goudeau 1976, Goudeau and Lachaise 1983, Glas et al. 1997). Recent studies, focusing primarily on the ultrastructural aspect of exoskeletal cuticle differentiation during embryonic development, refer mostly to the insect cuticle (Konopova and Zrzavy 2005, Moussian et al. 2006, Moussian 2010), and were performed also in the aquatic amphipod *Parhyale hawaiensis* (Havemann et al. 2008) and in *Porcellio scaber* (Mrak et al. 2014). As revealed in both these studies, the early extracellular matrix of epidermal cells appears as a thin delicate sheet of material. Later, the embryonic epidermis secretes a substantial matrix, termed embryonic cuticle or precuticular matrix, which is structurally different from the crustacean exoskeletal cuticle.

A cuticular matrix with the general characteristics of crustacean exoskeletal cuticle is formed in the last stages of embryonic development. The new cuticle formation in the subsequent marsupial manca stages and the renewal of the exoskeleton in late-stage marsupial mancae have been described by Mrak et al. (2012, 2014). Larvae, termed postmarsupial mancae, are released from the marsupium and develop in the external environment until the juvenile stage (Tomescu and Craciun 1987, Brum and Araujo 2007, Montesanto et al. 2012).

The structure, composition and formation of the cuticle in the digestive system of crustaceans have not been precisely characterized. The digestive system of isopod crustaceans consists of the ectodermal foregut and hindgut and endodermal digestive glands, named also hepatopancreas or midgut glands (Hames and Hopkin 1989, Wägele 1992, Štrus et al. 1995). The two main regions of the foregut are the esophagus and stomach, the latter termed also the proventriculus. In the hindgut three morphologically and functionally distinct sections are distinguished: anterior chamber, papillate region and rectum. A short midgut situated between the foregut and hindgut, connected to the hepatopancreas, was described in amphibious species of the family Ligiidae (Štrus and Drašlar 1988, Štrus et al. 1995). The ectodermal digestive tract epithelium is apically lined by cuticle and performs specific functions in certain gut regions, including grinding, filtration, transport and absorption of food, and ion transport (Hryniewiecka-Szyfter and Storch 1986, Storch 1987, Storch and Štrus 1989). In the early studies of the digestive tract epithelium in isopods, two layers of the gut cuticle were distinguished, characterized as epicuticle and endocuticle (Vernon et al. 1974, Palackal et al. 1984) and cuticular spines were observed, covering the majority of the gut surface (Storch and Štrus 1989). In the stomach of terrestrial isopods complex cuticular structures were described, forming elaborate masticatory and filtering devices (Storch and Štrus 1989).

Differentiation of the gut cuticle during embryonic development is a poorly understood issue. Embryos of terrestrial isopods develop in the aqueous environment of the marsupium, a fluid-filled brood pouch on the ventral side of the female body. Intramarsupial development of *P. scaber* lasts about 35 days under laboratory conditions and includes embryonic development, from fertilized egg to the early-stage embryo, the mid-stage embryo and the late-stage embryo, and development of the marsupial larva manca until release to the external environment (Milatovič et al. 2010). Wolff (2009) and Milatovič et al. (2010) have defined a staging system, describing twenty developmental stages, based on morphological characteristics of embryos and marsupial mancae in *Porcellio scaber*. Concerning digestive system development in embryos and marsupial mancae, the following morphological features of ectodermal digestive tract formation have been reported: (i) the invaginated stomodeum is discernible and hindgut invagination is evident between pleomere 6 and telson in mid-stage embryos (stage 6), (ii) the foregut and hindgut are fused in late embryos (stage 16), (iii) the cuticular masticatory apparatus, primary and secondary filters are present in the stomach of late embryos (stage 18), (iv) the hindgut is clearly partitioned into the anterior chamber and papillate region in late embryos (stage 18) and (v) the alimentary canal with

a pronounced typhlosole is fully developed in marsupial mancae (Štrus et al. 2008, Milatovič et al. 2010). Cuticle formation in the digestive system was not followed in detail in these studies.

In our study, ultrastructure of the hindgut cuticular lining in *P. scaber* embryos and marsupial mancae was characterized and compared to the hindgut cuticular lining of adults. We report on the hindgut cuticle differentiation from the structural viewpoint and discuss the results with respect to the differentiation of exoskeletal cuticle during intramarsupial development. Our aim was to describe the details of cuticle formation in different embryonic stages and to establish whether the hindgut cuticle of the emerging mancae is already fully developed.

Methods

Specimens of *Porcellio scaber* Latreille, 1804 (Crustacea: Isopoda) were maintained and bred in a laboratory culture, in soil and leaf litter, at 25 °C, high relative humidity and at 12-h light/12-h dark cycle. Three adult animals without any external signs of molting were selected from the culture and anaesthetized by cooling. The guts were isolated, rinsed in physiological solution (0.9% NaCl), cleaned and divided transversely into three portions. Samples were fixed in 2.5% glutaraldehyde in 0.1 M Hepes buffer (pH 7.2). After washing with 0.1 M Hepes buffer, the samples were postfixed in 1% osmium tetroxide for 2 h and washed again in the buffer.

Embryos and mancae were isolated from the marsupia of gravid *P. scaber* females and the stages of embryos were identified according to the existing staging system (Milatovič et al. 2010). The marsupial mancae were classified in accordance with the staging system reported by Mrak et al. (2012), distinguishing three sequential marsupial manca stages. In this study, three stages of late embryos (stage 16, stage 18 and stage 19) and two stages of marsupial mancae (early marsupial manca and late marsupial manca) were analysed. Specimens of embryos and mancae were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Prior to fixation, the egg envelopes of embryos were either carefully perforated with a thin needle or removed. After fixation, specimens were washed with the buffer, postfixed in 1% osmium tetroxide for 2 h and washed again in the buffer.

All samples were then dehydrated using ethanol and/or absolute acetone and were embedded in Agar 100 or Spurr's resin. Polymerization of the resin was performed at 60 °C for 48 h in embedding molds. Semithin and ultrathin sections were made with a Reichert Ultracut S ultramicrotome (Leica), using a glass or a diamond knife, respectively. Semithin sections were stained with Azure II – Methylene Blue and imaged with a Zeiss AxioImager Z.1 light microscope, equipped with a HRC Axiocam camera using Axiovision software. Ultrathin sections were contrasted with 4% uranyl acetate and 10% lead citrate and examined with a Philips CM100 transmission electron microscope. Images were recorded with BioScan 792 and Orius 200 (Gatan) cameras using Digital Micrograph software.

Results

Cuticular lining in the hindgut of adults

The hindgut epithelium consists of large cells, apically covered with cuticular matrix and subtended by muscle cells (Fig. 1). In the anterior chamber the apical parts of epithelial cells bulge into the gut lumen, while in the papillate region the cells protrude basally into the hemocoel, which accounts for the 'papillate' morphology of this region (Fig. 1A, B). The hindgut cuticle of adults is approximately 2 μm thick and consists of an electron dense epicuticle and an electron lucent procuticle (Fig. 1C–F). The major part of the epicuticle consists of a homogenous electron dense matrix. As revealed by detailed ultrastructure, two to three thin sublayers are discernible in the outermost part of the epicuticle (Fig. 1D inset). In the anterior chamber a thin layer of medium density is apparent between the epicuticle and procuticle (Fig. 1C, D). The procuticle in the anterior chamber is much thicker than the epicuticle, with the ratio of the thicknesses around 5:1. The procuticle displays ''lamellae'', appearing due to helicoidal arrangement of chitin-protein fibers (Fig. 1C). Short cuticular spines are present on the cuticle surface (Fig. 1D). In the papillate region of the hindgut, the thickness of epicuticle is similar to that of the procuticle and no pattern of the chitin-protein fibers arrangement can be discerned in the procuticle (Fig. 1E, F). In both hindgut regions the apical plasma membrane of epithelial cells is differentiated in a distinctive apical labyrinth, with abundant mitochondria apposed to the membranes (Fig. 1C, E).

Apical matrix of the hindgut cells in late embryos

The apical matrix of the hindgut cells in stage 16 late embryos consists of intensely ruffled electron dense lamina and more lucent homogenous material underneath. The apical surface of the epithelial cells frequently forms irregularly arranged membrane protrusions with electron dense plaques (Fig. 2A).

In embryos of the stage 18 the hindgut apical matrix consists of an intensely ruffled thin lamina on the surface and matrix underneath, which is more dense in comparison to stage 16 embryos (Fig. 2B, C, D). The surface lamina is three-layered, consisting of two electron dense sheets with a lucent sheet between them (Fig. 2B inset). The matrix under the lamina displays two regions: a distal region of medium density, and a proximal lucent region. Apical plasma membrane forms regularly arranged shallow protrusions with electron dense apical plaques. Closely apposed to these protrusions is another electron dense lamina, evident all along the hindgut, reflecting secretion of the new cuticle. The new lamina does not show any distinct substructure (Fig. 2D). It is formed from fragments, which are observed in some regions (Fig. 2C), but it is mostly evident as a continuous layer along the hindgut in this stage (Fig. 2B, D). Spine-like surface structures were not apparent.

In prehatching late embryos of stage 19, the apical matrix does not display a ruffled outline, but in general follows the apical gut surface. In some regions detachment

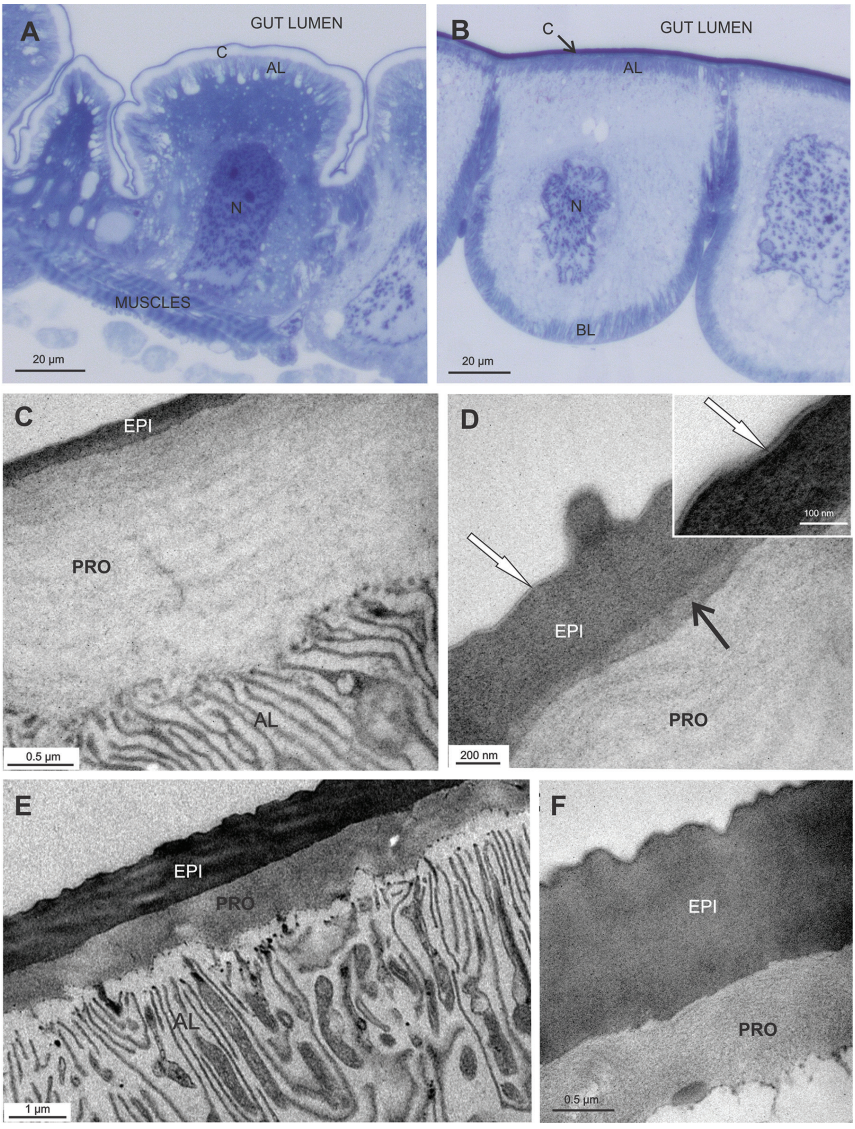

Figure 1. Hindgut epithelium and cuticle in *P. scaber* adults. **A** Semithin section of the hindgut anterior chamber. Gut cells protrude apically into the gut lumen. The apical membrane forms an apical labyrinth (AL), that is covered with the cuticle (C). N – nucleus of gut cell **B** Semithin section of the hindgut papillate region. Gut cells bulge basally into the hemocoel. Apical and basal labyrinths (AL, BL) are evident. Cuticle covers apical cell surface (C). N – nucleus of gut cell **C, D** Ultrastructure of the cuticle in anterior chamber. The cuticle is composed of thin electron dense epicuticle (EPI) and much thicker "lamellated" electron lucent procuticle (PRO). Several thin sublayers are discernible in the outermost part of the epicuticle (**D** inset - white →). A layer of medium electron density is visible between the epi- and procuticle (**D** - black →). A cuticular spine is present on the cuticle surface **E, F** Ultrastructure of the gut cuticle in papillate region. Epicuticle (EPI) and procuticle (PRO) are about the same thickness. Both are composed of morphologically homogenous matrix. Abundant mitochondria are observed closely to the membranes of the apical labyrinth (AL) **F** Several thin sublayers in the outermost region of the epicuticle are visible.

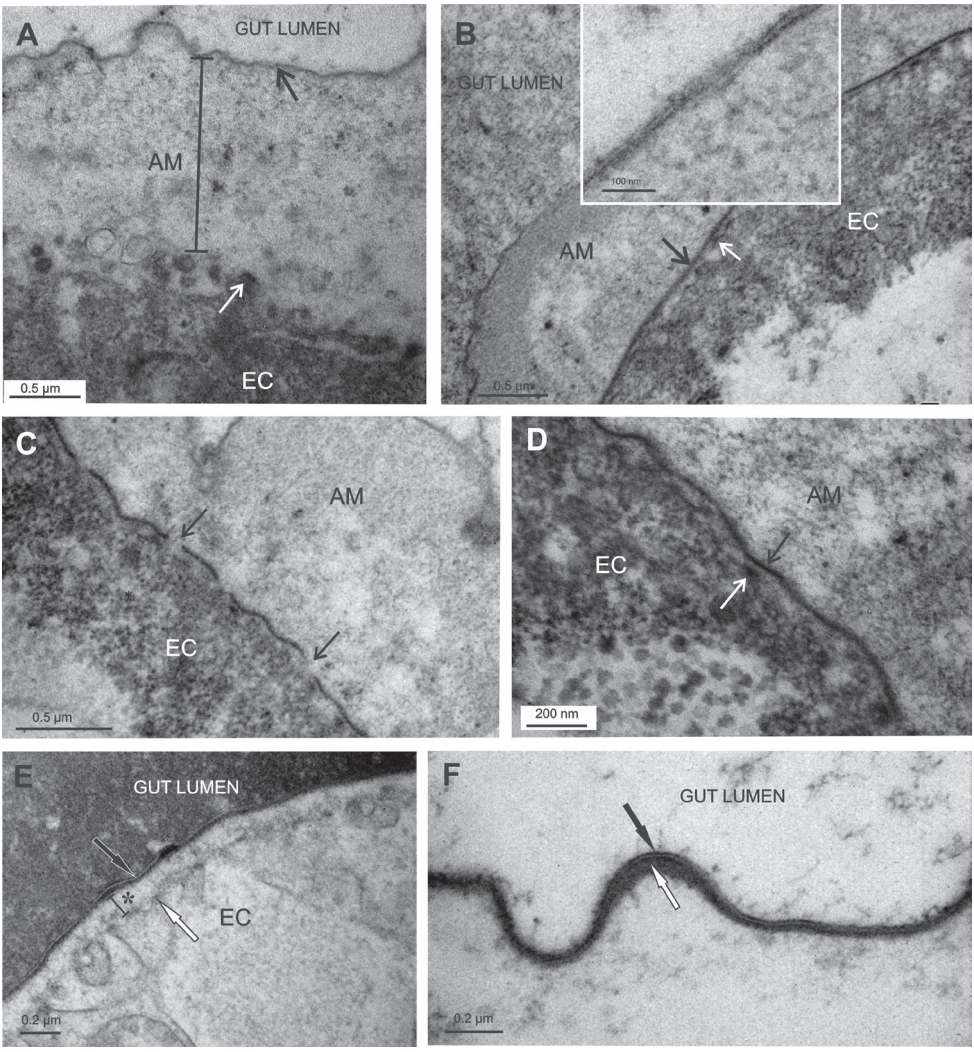

Figure 2. Apical matrices in the hindgut of *P. scaber* late embryos. EC - epithelial cell **A** The hindgut cells (EC) in stage 16 embryos are covered by a substantial apical matrix with intensely ruffled surface (AM). The matrix consists of an electron dense lamina (black →) and underlying more electron lucent homogenous material. The apical membrane displays irregularly arranged protrusions (white →) **B, C, D** In the stage 18 embryos the apical matrix of the hindgut (AM) is extensive. The surface lamina covers the matrix, which displays a distal region of medium density and a proximal lucent region. The lamina of this matrix is trilayered (**B** inset). A new electron dense lamina (**B, D** - black →) is evident above the apical membrane protrusions (**B, D** - white →). The new lamina is mostly continuous, though in some regions it still appears in fragments (**C** - black →) **E, F** In the prehatching embryo of stage 19 the hindgut apical matrix consists of a distal trilayered lamina (black →), an electron dense material, accumulating underneath the lamina (**F** - white →) and underlying lucent material (**E** - *****). Microvilli-like protrusions of the apical plasma membrane are evident (**E** - white →). The gut lumen is filled with homogenous material.

of the matrix from the epithelium was observed. The hindgut matrix in this stage is composed of an electron dense lamina facing the hindgut lumen and underlying lucent material. The lamina is thicker in this stage than in the previous embryonic stages and consists of three layers (Fig. 2E, F). In addition, electron dense material of about 20 nm in thickness is accumulated underneath the lamina. This accumulation, seen in the cuticular matrix of the hindgut in adults, is first observed in this stage. The apical plasma membrane of the hindgut cells forms microvilli-like protrusions (Fig. 2E).

Cuticular matrix in the hindgut of marsupial mancae

The luminal side of the hindgut epithelium of marsupial mancae is covered with cuticle, aligned to the surface of cells (Fig. 3) and consisting of epicuticle with a thin trilayered lamina and electron dense material underneath and procuticle, a thick homogenous lucent layer (Fig. 3B–E). No pattern of helicoidally arranged chitin-protein fibers is discernible in the procuticle. The apical plasma membrane is intensely invaginated, forming a labyrinth (Fig. 3A–E).

In early marsupial mancae the electron dense material under the trilayered lamina of the epicuticle is slightly more abundant than in stage 19 prehatching embryo (Fig. 3B, C). In some regions the cuticle protrudes into the gut lumen and electron dense material often accumulates in this bulge (Fig. 3A, B). In late marsupial mancae the electron dense material of the epicuticle is around 50 nm thick, which is slightly thicker than that in earlier larvae but still considerably thinner than in the cuticle of adults. Cuticular spines are evident on the cuticle surface in both the anterior chamber and the papillate region (Fig. 3D, E). The apical plasma membrane forms a prominent apical labyrinth (Fig. 3D, E). In addition, the morphological characteristics of cuticle renewal are observed in some specimens (Fig. 3F). Following apolysis, the old cuticle is detached from the epithelium and partly degraded, particularly in the basal part. In the formed ecdysial space the new cuticle formation takes place on the apical plasma membrane protrusions (Fig. 3F, 3F inset). The new cuticle is composed of two distinctive layers of similar thickness, the electron dense epicuticle and the inner homogenous procuticle (Fig. 3F).

In some examined specimens the gut lumen was empty, although in most marsupial mancae homogenous gut contents were observed (Fig. 4). In one specimen of the early marsupial manca, numerous bacteria were evident within the homogenous contents of the hindgut lumen (Fig. 4A, B). These bacteria have an electron dense cytoplasm, are rod-shaped, about 0.5 μm × 1.5–2 μm in size, and always surrounded by lucent spaces. The hindgut epithelial cells of the late marsupial manca contain lipid droplets, which are accumulated mostly in the basal part of the cytoplasm and are more abundant in the ventral gut epithelium (Fig. 4C, D). Differences in hindgut cell shapes are also observed between the dorsal and ventral epithelia, with the ventral cells being prismatic and apically bulging into the lumen and the dorsal cells by the typhlosole showing a more isodiametric shape (Fig. 4C).

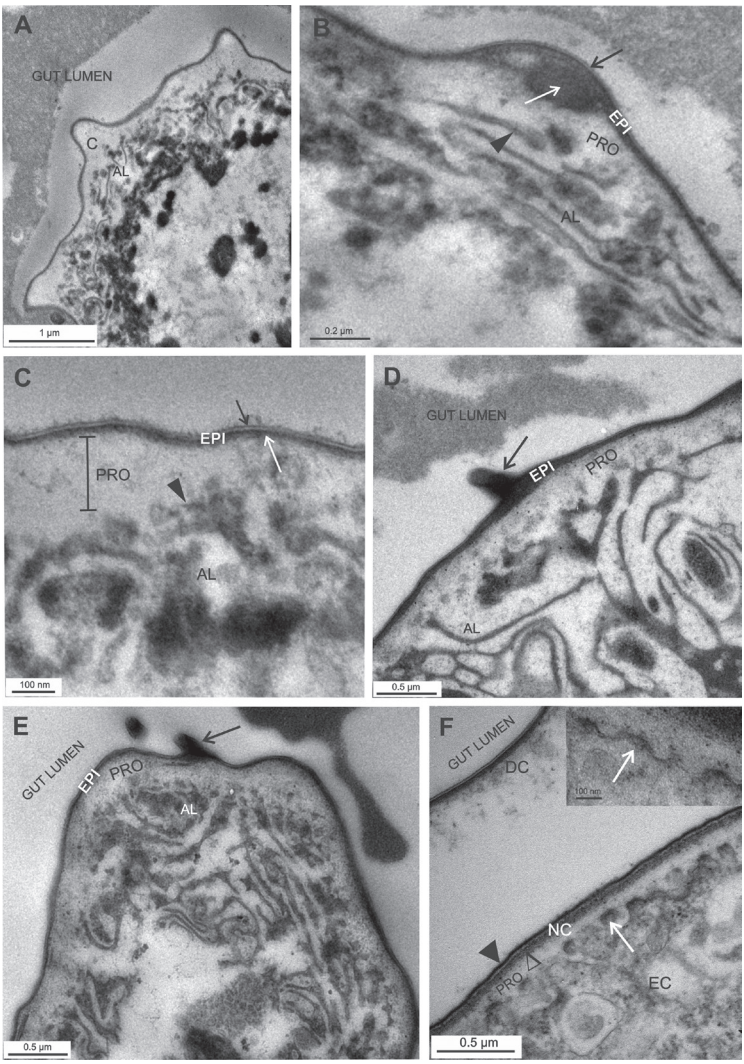

Figure 3. Cuticle in the hindgut of *P. scaber* marsupial mancae. EC - epithelial cell, PRO – procuticle, EPI – epicuticle, AL – apical labyrinth. **A, B, C** The hindgut cuticle (**C**) in early marsupial manca with the outer epicuticle and the inner procuticle. The epicuticle (EPI) consists of the outermost trilayered lamina (**B, C** - black →) and electron dense material underneath (**C** - white →). The procuticle (PRO) contains homogenous electron lucent material. Bulges of the cuticle are observed, some include electron dense material (**B** white →). Apical plasma membrane is intensely invaginated (**B, C** – ►) and forms apical labyrinth (AL) **D, E** The hindgut cuticle in late marsupial manca in the anterior chamber (**D**) and in the papillate region (**E**). Electron dense material is prominent under the trilayered lamina of the epicuticle. Cuticular spines are evident (black →) **F** Hindgut cuticle renewal in late marsupial manca - degradation and detachment of the old cuticle (DC) and formation of the new cuticle (NC) on the plasma membrane protrusions (white →). The new cuticle consists of an electron dense lamina (►), an electron dense material accumulating underneath (Δ) and an inner electron lucent homogenous procuticle (PRO) **F** inset: Protrusions of the apical plasma membrane (white →) display electron dense tips – plaques – and are covered with an electron dense material.

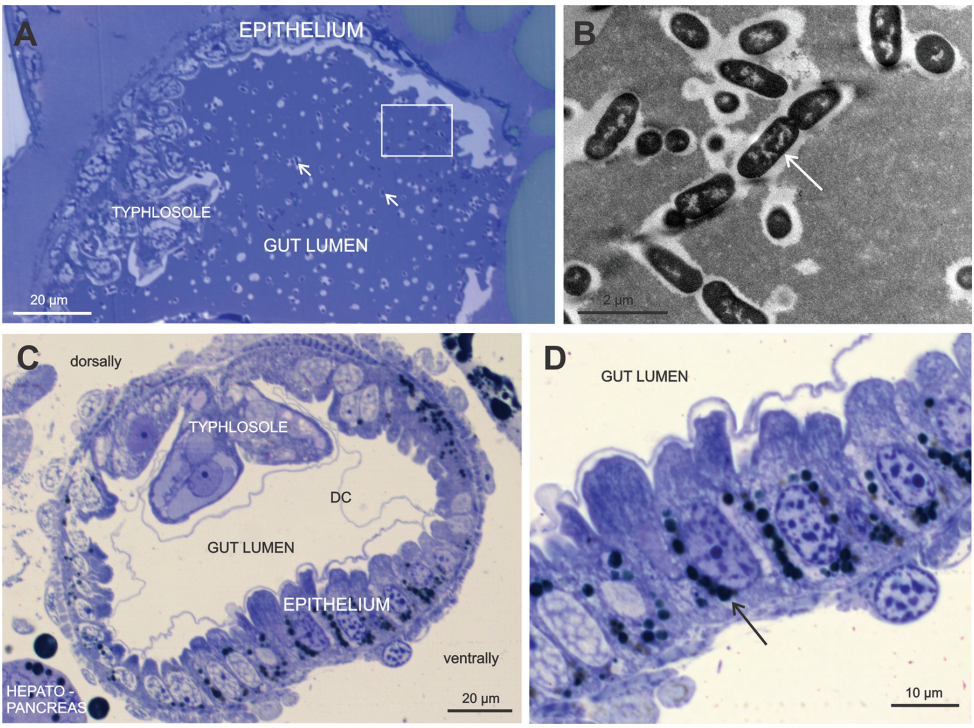

Figure 4. A, B The gut lumen contents in the early marsupial manca of *P. scaber* includes homogenous material with evenly distributed bacteria (white →). A higher magnification of the squared area in the image **A** is shown in the image **B** Bacteria are rod-shaped, contain electron dense cytoplasm and are surrounded by lucent spaces. **C, D** Empty gut lumen, observed in the late marsupial manca. The cuticle is in most regions considerably detached from the epithelium (DC). The epithelial cells are ventrally more prismatic and dorsally more isodiametric. A higher magnification of the ventral gut cells in the image **D** reveals basally accumulated lipid droplets (black →).

Discussion

Morphogenesis of the digestive system was previously studied in isopod crustacean *Porcellio scaber* (Štrus et al. 2008, Milatovič et al. 2010). Development of the cuticular lining in the ectodermal digestive tract is also essential for gut function, but gut cuticle formation was not studied in detail. In this study the hindgut cuticle differentiation in *P. scaber* embryos and marsupial mancae is described and discussed with respect to the hindgut cuticular lining in adults and with respect to exoskeletal cuticle differentiation.

The hindgut cuticular lining in adults of *P. scaber* is approximately ten times thinner than the exoskeletal cuticle. We show here that in the anterior chamber of the hindgut the procuticle is much thicker than the epicuticle, the ratio of the thicknesses being approximately 5:1. In the hindgut papillate region the epicuticle is about the same thickness as the procuticle and several times thicker than the epicuticle in the anterior chamber. The procuticle of the anterior chamber displays ''lamellae'' similar

to those in the exoskeletal cuticle that appear due to the helicoidally arranged chitin-protein fibers. In contrast, the procuticle in the papillate region is morphologically homogenous. Two hindgut regions, anterior chamber and papillate region, are known to perform specific functions (Hames and Hopkin 1989) and are also characterized by specific ultrastructural features of the gut cuticle, as we show here.

The apical matrix secreted by hindgut cells in late embryos of stages 16 and 18 consists of loose material overlaid by an intensely ruffled electron dense lamina and thus resembles the epidermal precuticular matrix that is formed prior to exoskeletal cuticle during embryonic development (Mrak et al. 2014). Thickness of this hindgut matrix is in the same range as that of the epidermal precuticular matrix. Hindgut and epidermal matrices that are structurally similar in these embryonic stages, differentiate during further development into two cuticles of considerably different structure and thickness. The structural resemblance of embryonic gut and epidermal precuticular matrices in this species to the embryonic epidermal matrices preceding cuticle formation in insects and other crustaceans (Goudeau 1976, Goudeau and Lachaise 1983, Glas et al. 1997, Moussian et al. 2006, Konopova and Zrzavy 2005, Havemann et al. 2008) implies a more common principle in differentiation of chitinous matrices. The embryonic gut matrix is structurally different from the gut cuticle in adults, indicating that it does not yet perform all the specific functions of the fully differentiated gut cuticle in the adults. Still, this matrix may function as a protective barrier or may participate in a transport regulation. The intensely ruffled surface and loose structure suggest that the gut matrix in late embryos may serve to accommodate changes related to growth and bending of the embryo. In addition, the connection of the embryo with the osmoregulatory dorsal organ is lost at this period of embryogenesis, and this probably causes changes in the osmoregulatory capacity of the embryo (Milatovič et al. 2010).

The hindgut matrix in prehatching stage 19 embryos consists of a trilayered electron dense lamina, subjacent electron dense material and the innermost lucent layer. We consider this matrix a hindgut cuticle as it strictly follows the apical epithelial surface and includes a prominent layer of electron dense material below the lamina, characterizing the gut epicuticle. The apical membrane protrusions in some regions suggest the secretion of the procuticle components. In this stage the procuticle in the hindgut is not sublayered as it is in fully formed cuticle, while the exoskeletal cuticle already displays ultrastructure similar to that in adults (Mrak et al. 2014). As an electron dense lamina apposed to the apical membrane of the hindgut cells was observed in the preceding developmental stage – stage 18 embryo – we consider this the first epicuticle formation. The results thus indicate that the cuticle is secreted prior to the shedding of the early apical matrix. The lamina is still discontinuous in some regions and does not form any surface structures. In this developmental stage (stage 18) the first exoskeletal cuticle formation has been described and observed in all regions as a continuous cuticular matrix, also forming epicuticular scales (Mrak et al. 2014). The early phase of exoskeletal cuticle formation during embryogenesis of *Drosophila melanogaster* has been characterized by the presence of fragments of the outer epicuticle, which is described in insects as an ''envelope''. In later development the gaps between

the fragments are closed, forming a continuous envelope (Moussian et al. 2006). Our results indicate that the initiation of hindgut cuticle formation similarly appears in fragments, which is consistent with exoskeletal cuticle formation described in other arthropods, for example in *D. melanogaster* (Moussian et al. 2006).

The hindgut cuticular lining in marsupial manca, with the electron lucent procuticle and overlying electron dense epicuticle, shows more similarities to the hindgut cuticle in adults. Advanced differentiation is evidenced by more prominent electron dense layer of the epicuticle and formation of cuticular spines. Extensive invaginations of the apical plasma membrane, forming a prominent apical labyrinth, suggest that hindgut epithelium is involved in transportive processes in mancae. The morphology of hindgut epithelium and cuticle in marsupial mancae implies that the specific functions of the hindgut in feeding are more developed. We have observed that in most examined mancae the gut lumen is filled with homogenous contents. Warburg (1994) also reports on intramarsupial cannibalism of marsupial mancae. Compared to the hindgut cuticle of adults, the cuticle in marsupial manca is five to twenty times thinner, the procuticle in the anterior chamber is not sublayered and the ratio of epicuticle to procuticle thicknesses is similar in different hindgut regions. These differences indicate that the hindgut cuticle in marsupial mancae does still not perform all functions of the gut cuticle in adults, which could be related to the aqueous environment of the marsupium, among other not exposing the animal to dissication. As described for the exoskeleton (Mrak et al. 2012, 2014), morphological characteristics of cuticle renewal in late marsupial mancae are observed also in the hindgut, indicating the renewal of the gut cuticle soon after release of mancae from the marsupium and subsequent molting of the mancae. Bacteria recorded in one specimen of an early marsupial manca were distributed evenly within the contents of the hindgut lumen and no specific attachments to the cuticular lining were observed. Kostanjšek et al. (2003) reported on autochthonous bacteria associated with the wall of the hindgut papillate region in adult *P. scaber*. They described rod-like bacteria of 2.5 μm ± 1 μm in length and 200 nm ± 50 nm in width and filamentous microbes with lengths up to 6 μm, both specifically attached to the cuticular spines. Bacteria observed in our study are not attached to the hindgut cuticle and resemble neither the rod-like bacteria by size nor the filamentous microbes described by Kostanjšek et al. (2003).

Conclusions

Ultrastructural characteristics of the hindgut cuticular lining and epithelium, according to the examined developmental stages of *P. scaber* and in comparison to the hindgut of adults, are shown in a schematic representation in Figure 5. Identified by ultrastructural analysis, the early stages of hindgut apical matrix formation during embryonic development are similar to the early differentiation of epidermal matrices, reported in arthropods. This structural resemblance suggest similar underlying processes of chitinous cuticle differentiation. Structural difference from the hindgut cuticle in adults

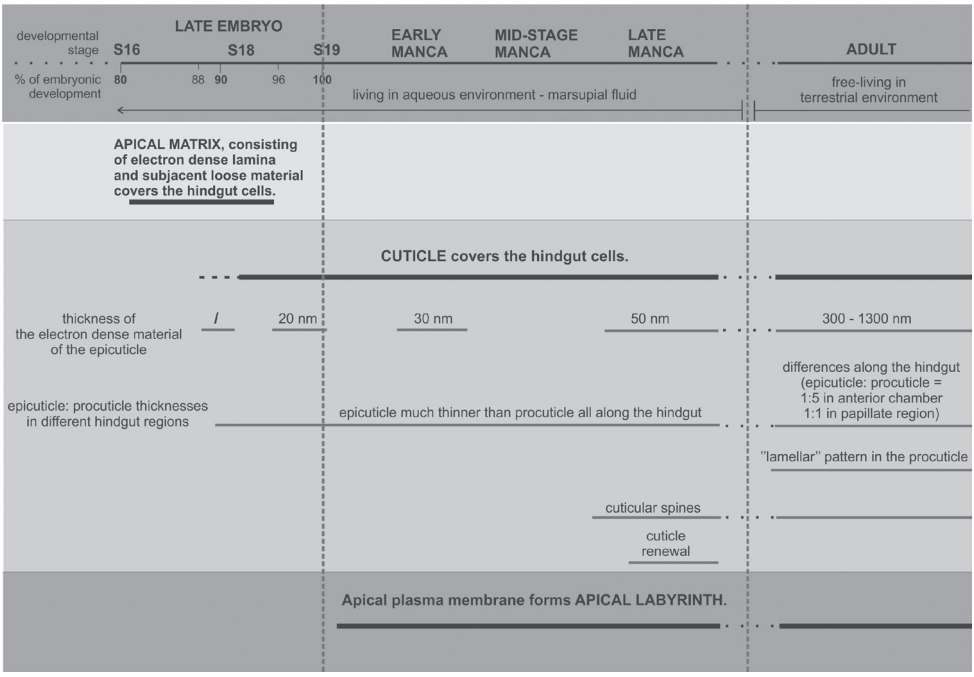

Figure 5. A schematic representation showing the ultrastructural characteristics of the hindgut apical matrices and epithelium during late intramarsupial development and in comparison to the hindgut cuticular lining of adult animals in *P. scaber*. The axis represents the successive developmental stages and the percentage of embryonic development. The vertical dashed lines indicate the transition from embryonic to larval development and from larval development to adult stage. The thick horizontal lines represent presence of the individual feature in the certain stages. The specific features of the cuticle are indicated by the thin lines.

suggests that early hindgut apical matrices are not fully involved in the specialized functions of the cuticle.

In the advanced intramarsupial stages the hindgut cuticle is structurally more similar to the hindgut cuticle in adults, suggesting it progressively assumes its specific functions in protection and food processing. In the stage 19 prehatching embryo a hindgut cuticle is evident, characterized by alignment to the epithelial surface and a prominent electron dense layer of the epicuticle. An electron dense lamina in the preceding embryonic stage - stage 18 - is evident, considered as the first epicuticle formation. This is the same stage, in which the first exoskeletal cuticle formation has been observed.

In marsupial mancae further gut cuticle differentiation is evident, as formation of the cuticular spines and a conspicuous electron dense epicuticular layer. Compared to the gut cuticular lining of adults, the cuticle of marsupial mancae is thinner, the ultrastructure is not different along the length of the hindgut, the procuticle in the anterior chamber does not yet show any lamellae associated with chitin helicoids, and the electron dense layer of the epicuticle is thinner. These differences imply that the function of the

hindgut cuticle in marsupial manca is not fully developed, possibly related also to different environments, as mancae develop in marsupial fluid. Synthesis of new cuticle prior to ecdysis is evident in both the exoskeleton and hindgut cuticle of late marsupial mancae.

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RESEARCH ARTICLE

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Spectroscopic parameters of the cuticle and ethanol extracts of the fluorescent cave isopod *Mesoniscus graniger* (Isopoda, Oniscidea)

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Abstract

The body surface of the terrestrial isopod *Mesoniscus graniger* (Frivaldsky, 1863) showed blue autofluorescence under UV light (330–385 nm), using epifluorescence microscopy and also in living individuals under a UV lamp with excitation light of 365 nm. Some morphological cuticular structures expressed a more intense autofluorescence than other body parts. For this reason, only the cuticle was analyzed. The parameters of autofluorescence were investigated using spectroscopic methods (molecular spectroscopy in infrared, ultraviolet-visible, fluorescence, and X-ray fluorescence spectroscopy) in samples of two subspecies of *M. graniger* preserved in ethanol. Samples excited by UV light (from 350 to 380 nm) emitted blue light of wavelengths 419, 420, 441, 470 and 505 nm (solid phase) and 420, 435 and 463 (ethanol extract). The results showed that the autofluorescence observed from living individuals may be due to some β-carboline or coumarin derivatives, some crosslinking structures, dityrosine, or due to other compounds showing similar excitation-emission characteristics.

Keywords

Mesoniscus graniger, autofluorescence, molecular spectroscopy, β-carboline and coumarine derivatives

Introduction

Among arthropods, the fluorescence of body surface was firstly reported in scorpions. The intensity of the fluorescence increased with the hardening of the cuticle (Pavan and Vachon 1954, Lawrence 1954). However, other invertebrates, e.g. cockroaches (Neff et al. 2000) and marine as well as freshwater crustaceans (Zimmer et al. 2002, Mazel 2005, Haug et al. 2011) also showed fluorescence.

Scorpions emit visible light (400–700 nm) under UV radiation (Fasel et al. 1997). In *Euscorpius italicus* (Herbst, 1800) the fluorescent substance is concentrated in the thin hyaline layer of the cuticle and is insoluble in water below 100 °C as well as in other solvents such as ethyl ether, chloroform, acetone, benzene, toluene, and methanol (Pavan and Vachon 1954). However, the fluorescent substance may be partly soluble in alcohol, in which scorpions are preserved (Wankhede 2004).

Stachel et al. (1999) determined the soluble fluorescent compound from scorpion cuticle as an alkaloid β-carboline using separation by thin layer chromatography and compound identification by nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC). β- carboline was also reported from the human cataracts (Wankhede 2004). β-carboline derivatives, some with hallucinogenic effects, are known from some plants (Hadley et al. 1974, Cao et al. 2007).

It is assumed that more than one fluorescent compound may be present in scorpions. 7-hydroxy-4-methylcoumarin was detected as another fluorescent compound in an extract of scorpion cuticle by Frost et al. (2001) using HPLC for separation and detecting the fluorescence by fluorimetry. The substance was identified by gas-chromatography mass-spectrometry (GCMS). 7-hydroxy-4-methylcoumarin is often used as fluorogenic marker in enzyme assays (also known as 4-methylumbelliferone - Miller et al. 1998, Gee et al. 1999). Coumarin derivatives were found mainly in plants, but also in prosobranch molluscs and in the scent glands of beavers (Murray et al. 1982). Another possible fluorescent compound found in the cuticle of arthropods is resilin. It is a very elastic protein with an irregular structure: its randomly coiled chains are crosslinked by di- and tri-tyrosine links (Elvin et al. 2005). In the cockroach *Periplaneta americana* (Linnaeus, 1758) fluorescence of the ligaments of the tarsus containing resilin was observed (Neff et al. 2000).

The autofluorescence of the cuticle of the cave isopod *Mesoniscus graniger* (Frivaldsky, 1863) was found during analysis of the content of its digestive tract under fluorescent microscope (Giurginca et al. 2012). *M. graniger* is the first terrestrial isopod in which autofluorescence was observed from the entire body. Autofluorescence was recorded in the isopod *Nataldillo burnupi* (Collinge, 1917) by Lawrence (1954) but only a weak one from the sternites; the chemical compound responsible for this isopod autofluorescence is not yet known. The aim of our study was therefore, to describe the autofluorescence in detail using microscopic observations and to measure spectroscopic characteristics of the substances responsible for the *M. graniger* cuticle autofluorescence. Only the cuticle was investigated; although in the ethanol extracts, there might be fluorophore products

resulting from the dissolution of the soft tissues, in our opinion the cuticle (the exoskeleton) contributed the most of the fluorescent signal. Moreover, the cuticle of *Mesoniscus* has not enough transparency to allow the observation of the soft tissues fluorescence.

Material and methods

Material

Living as well as individuals of *M. graniger* preserved in ethanol, were used in our study. Living animals were sampled for epifluorescent microscopy in the Slovak Karst National Park (Domica and Ardovská caves). The individuals stored in ethanol used for spectroscopic analyses were collected in the Romanian Karst: the Cernişoara Valley, 20 individuals corresponding to the subspecies *M. graniger graniger* (Frivaldsky, 1863) (labelled in the following analyses as G) and from the Sighiştelului Valley, 16 individuals corresponding to the subspecies *M. graniger dragani* Giurginca, 2003 (labelled in the following analyses D). In order to assess if the autofluorescence is present in the entire range of *M. graniger*, we used individuals from the Petnička Pećina (Valjevo, Serbia) and for assessing the presence of this feature in both species of the genus *Mesoniscus*, we tested the individuals of *Mesoniscus alpicola* (Heller, 1858) from the Falkensteinhöhle (Niederösterreich, Austria).

Fluorescence imaging

Photographs of living fluorescent individuals of *M. graniger* were recorded with the Olympus XZ61 stereomicroscope equipped with the Olympus DP20 camera and the Hoya UV (0) photographic filter using the Helling UV-Inspector 385 lamp (365 nm) as a source of excitation light. Animals were placed in a refrigerator for a minute to reduce their movement before taking pictures. Images obtained in different focal planes were stacked by the Helicon Focus 5.3 software (Helicon Soft, Ltd.) to obtain a large depth of focus for the resulting photos. Details of fluorescent body surface of *M. graniger* were documented on the Olympus BX 60 fluorescent microscope equipped with the Olympus DP50 camera. The Olympus U-MWU mirror unit (330-385 nm exciter filter and BA420 barrier filter) was used.

Under field conditions, the autofluorescence of living animals was documented with the Canon EOS 600D camera under the excitation light of the Helling UV-Inspector 385 lamp in the Ardovská Cave (Slovakia).

The autofluorescence of *M. graniger* from Serbia and that of *M. alpicola* was confirmed under the Bactericide Lamp LBA 55W (253.7 nm) and the First Light Illuminator-System Biodoc (302 nm). No spectral analyses were performed on the samples of *M. graniger* from Serbia and on the samples of *M. alpicola*.

Spectroscopic analyses

Sample preparation:
The samples preserved in 75% ethanol were filtered in order to separate the solid from the liquid phase (ethanol extract). The solid phase was air dried and stored in Petri-type laboratory vessels; the liquid phase was kept in Erlenmayer-type laboratory vessels.

Apparatus and investigation methods:
For the analyses of samples (G solid phase, G ethanol extract, D solid phase, and D ethanol extract) we used molecular spectroscopy techniques in the infrared (IR) (middle – MID and near – NIR), ultraviolet-visible (UV-VIS) and fluorescence (FP) range. In addition, a part of each sample was analyzed by X-ray fluorescence spectroscopy (XRF).

For the **IR analysis**, we used the Bruker Optics Tensor 27 spectrometer, with Opus 4.2 specialized software, in the 500–4000 cm^{-1} range. The analysis used the spectral KBr technique with a device for micro-pellets. The IR analysis was used for the solid samples and the ethanol extracts.

For the **UV-VIS and NIR analysis**, we used the UV-VIS-NIR-620 apparatus (Jasco, Japan) with 10 ml quartz cells for the liquid phase and with the ILN-725 diffuse reflection accessory for the solid phase, in the 200–2500 nm range. The apparatus has a monochromator and photoelectric cells corresponding to the investigated domains (UV = 200–400 nm, VIS = 400–800 nm and NIR = 800–2000 nm). Although the NIR region is a part of the IR spectroscopy, for constructive reasons it was included in this apparatus, the energy source being more powerful than that used for the IR range. The UV-VIS and NIR analysis was used for the solid samples and the ethanol extracts.

For the **molecular fluorescence analysis**, we used the FP6500 and FP6300 spectrofluorimeters (Jasco, Japan) using 10 ml quartz cells for the liquid phase and special tanks with quartz window for the solid phase, in the 200–800 nm range. Specific wavelengths were used for excitation in the UV-VIS range, with sources specific to each spectral region (UV and VIS) and the emission spectra were registered. The FP analysis was used for the solid samples and the ethanol extracts.

For the **XRF (X-ray fluorescence) analysis**, a part of each sample was grounded in an agate mortar and, subsequently, loaded into small plastic cylinders and XRF-analyzed on a Horiba XGT-7000 X-ray Analytical Microscope. The XRF analysis was used only for the solid samples.

Results and discussion

Autofluorescence microscopy

Mesoniscus graniger body surface shows a blue auto-fluorescence when excited with UV light at a wavelength of 365 nm (Fig. 1a) or 330–385 nm (Fig. 1b). Tubercles on the

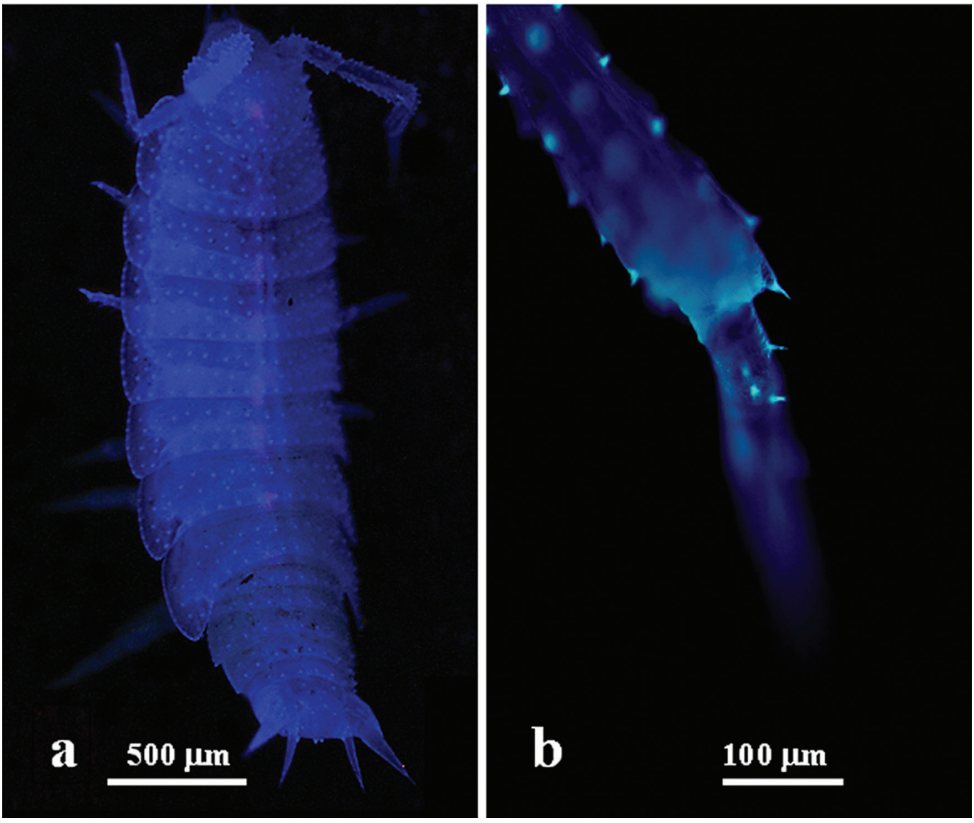

Figure 1. Autofluorescence of the body of *Mesoniscus graniger* under UV light. **a** stereomicroscope with UV-inspector 385 (excitation light 365nm) **b** detail of the antennae - fluorescence microscope U-MWU mirror unit (330–385 nm).

cuticle surface have a pale blue auto-fluorescence more intense than all the rest of the body surface (Fig. 1a, b).

Autofluorescence was present in all tested specimens of *M. graniger* collected from different localities inhabited by this species from the Slovak to the Serbian karst regions. Both subspecies of *M. graniger* from Romania (*M. graniger graniger* and *M. graniger dragani*) show the same intensity of autofluorescence, which is also found in *M. alpicola*, the second species of the genus.

Autofluorescence in the field

Following observations made under laboratory conditions, we tried to document the autofluorescence under field conditions (See Suppl. material 1). As the movie clearly shows, under visible light *Mesoniscus* presents a white color, but under UV light, the body surface shows a blue auto-fluorescence.

Molecular spectral analysis

The FT-IR (Fourier Transform Infrared) analysis of the solid phase of both subspecies (Fig. 2D, G) showed a polypeptide structure with characteristic bands at 1650 cm^{-1} (νC=O – amide I), 1542 cm^{-1} (δNH – amide II) and amide III (νC-N-C – 1240 cm^{-1}) besides aliphatic (νCH, νCH2) at 2955–2850 cm^{-1} and hydroxyl + amino groups (νOH + νNH) at 3405–3300 cm^{-1}, originating in the constitutive amino acids and the glucosamine (Fig. 2D, G) (Balaban et al. 1983). Other FT-IR spectra bands originate from $CaCO_3$ (1415 and 873 cm^{-1}). The 1113 and 1100 bands are resulting from C-OH and C-NH groups from the N-acetyl glucosamine (chitine) (Fig. 2D).

The FT-IR spectra of the sample of the subspecies *M. graniger graniger* (Fig. 2G) presented a series of peculiarities, in which the ageing of the sample must be taken into consideration. A higher content of $CaCO_3$ and changes in the peptide structure were recorded, explaining the differences in the spectra: a diminution of the 1650 and 1542 cm^{-1} bands (δNH – amide II) and the disappearance of the 1240 cm^{-1} band (amide III) pointing to the alteration of the polypeptide structure with the involvement of the NH group. Carbonyl/carboxyl structures at 1720 and 1800 cm^{-1} (ketones and/or organic acids), point to a hydrolytic type of oxidative process with the involvement of the NH

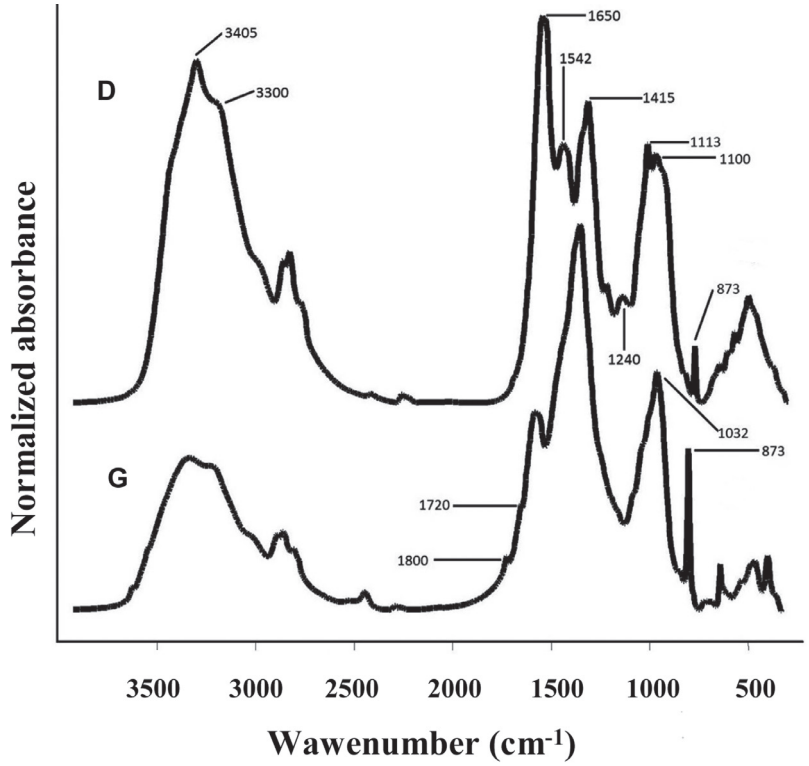

Figure 2. IR spectra of the samples of solid phase of *M. graniger graniger* (**G**) and *M. graniger dragani* (**D**).

group from N-acetyl-glucosamine highlighted by the 1032 cm^{-1} band, attributed to the N-CO-C group (Nenițescu 1965). There were no other differences between the bands recorded for the subspecies of *M. graniger*.

The FT-IR analysis of the ethanol extract of the subspecies *M. graniger graniger* showed bands belonging to aromatic fragments and some oxidation compounds (carbonyl group νC = O at 1725 cm^{-1}), pointing to a break in the amidic chain proved by the absence of the 1240 cm^{-1} band (amide III). The absence of the 1240 cm^{-1} band might be due to the insolubility of some compounds (Fig. 3).

The presence of Ca, already inferred by the IR analysis, was confirmed by the **XRF analysis**, the Ca content (weight %) being 43.83% in sample G and 16.25% in sample D (expressed as Ca^{2+}).

The analysis in the UV-VIS-NIR domains of the samples solid phase undertaken on the material of the subspecies *M. graniger dragani* only (D samples) showed several characteristic bands (Fig. 4): a wide band situated between 200 and 600 nm, with a maximum at 331 nm, pointing to a combination between the transitions $\pi \rightarrow \pi^*$ + $n \rightarrow \pi^*$ and an extended conjugation system (Balaban et al. 1983); the 1507 nm band emphasizing the presence of intra/intermolecular hydrogen bonds formed with the involvement of the OH and NH groups from peptides and chitin; the bands from 1732, 1946 and 2292 nm come from hydroxylic groups (νOH + δOH) present in the polypeptide chain, but also in chitin (Egawa et al. 2003, Badea et al. 2008).

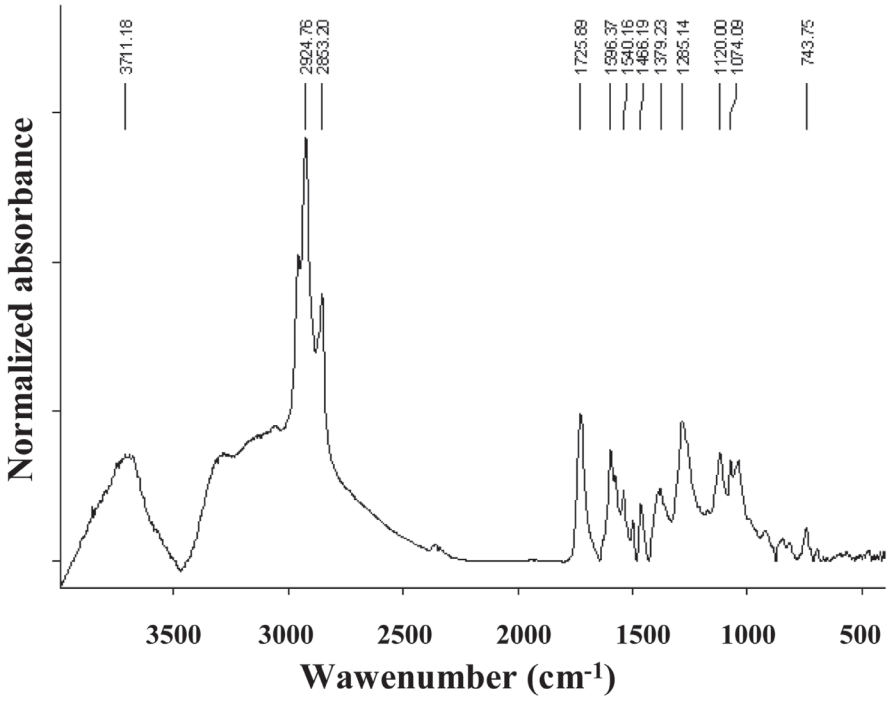

Figure 3. IR spectra of the ethanol extract of *M. graniger graniger*.

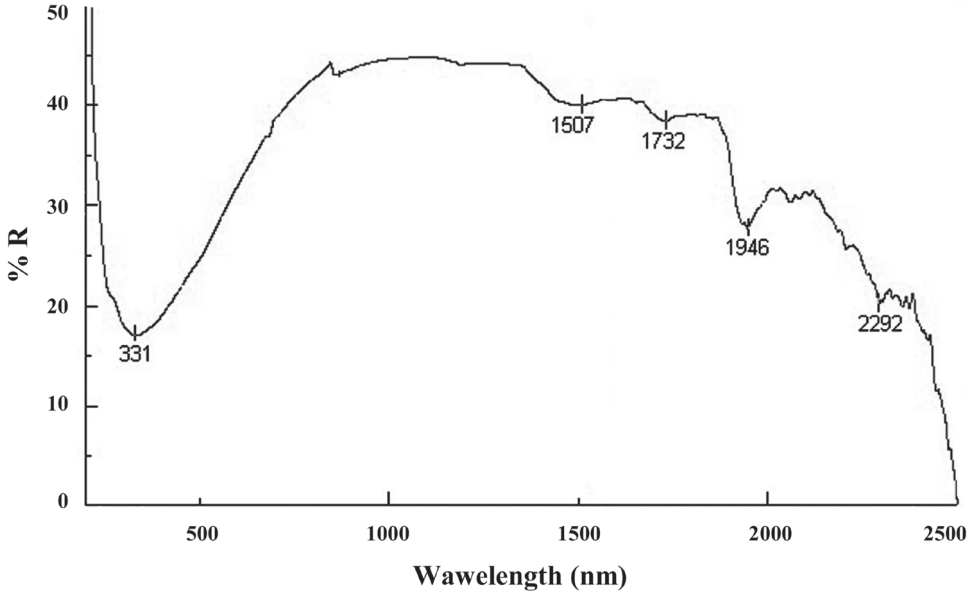

Figure 4. UV-VIS-NIR spectra of the sample of solid phase of *M. graniger dragani* (% R = percent reflectance).

The UV-VIS-NIR analysis of the ethanol extracts (Fig. 5D, G) presented only bands characteristic to the $\pi \rightarrow \pi^*$ + $n \rightarrow \pi^*$ transitions in the 210-220 nm belonging to the aromatic structures and $n \rightarrow \pi^*$ at 275-280 nm (the CONH group from amino acids). The D sample showed a weak band coming from conjugated structures which led to the yellow colour of the solution.

The molecular fluorescence analysis (FP) of the samples of solid phase of *M. graniger dragani* obtained by excitation at 265 nm (Fig. 6) showed several characteristic bands: the 280 nm band can be attributed to the phenylalanine (Lakowicz 2002); the 303 nm band was expressed due to the presence of tyrosine (Lakowicz 2002); the 417 and 440 nm bands are attributed to some crosslinking structures (possibly lipids from membranes) and to the dityrosine (Dolgin et al. 2009, Ross et al. 2002); the 469 band points to the presence of a β-carboline derivative, taking into account the light blue - blue colour of the fluorescent emission (Stachel et al. 1999).

The FP analysis with excitation at 380 nm (Fig. 7) led to the emission spectra with bands at 419 nm and 441 nm responsible to the crosslinking structures (containing bonds between molecular chains with the involvement of aromatic structures) and dityrosine, 470 nm to β-carboline derivative and 505 nm corresponding to the fluorophore structure with extended conjugation resulting more probably from lipid oxidation (Sokolov et al. 2002).

The molecular fluorescence analysis of the ethanol extracts were obtained by excitation at 280, 350 and 380 nm, the colour of the emission being light blue - blue (see Table 1).

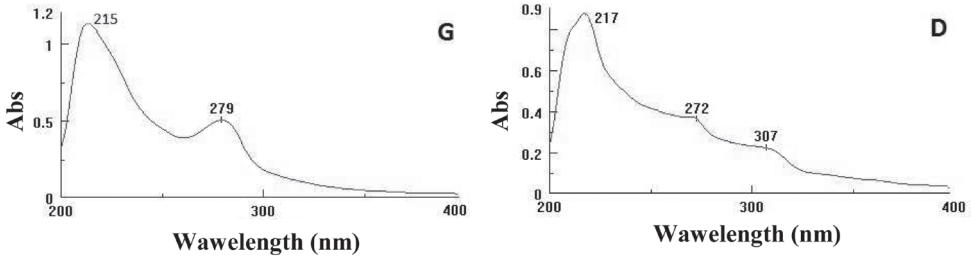

Figure 5. UV-VIS-NIR spectra of the ethanol extracts of *M. graniger graniger* (**G**) and *M. graniger dragani* (**D**) (Abs = Absorbance units).

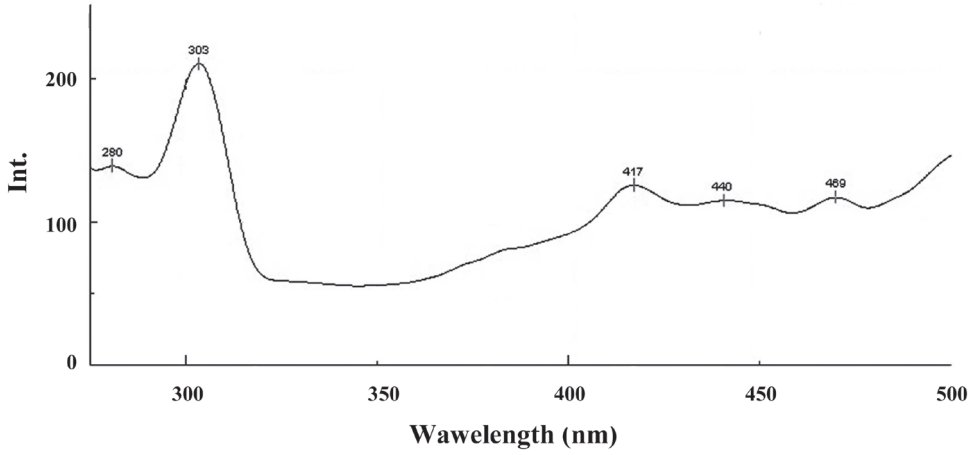

Figure 6. FP spectra of the sample of solid phase of *M. graniger dragani* (λex = 265 nm) (Int.- intensity of the peak).

Table 1. The emission bands of the ethanol extracts.

	D	G
λ excitation (nm)	λ emission (nm)	λ emission (nm)
280	317	313
350	420	435
380	435	463

The bands from 313 and 317 nm are attributed to the presence of tyrosine and some aromatic structures with hydroxyl groups (λex = 280 nm) (Ross et al. 2002). The bands from 420 and 435 nm result from crosslinking structures and/or the formation of dityrosine by intra/intermolecular hydrogen bonds (λex = 350 nm) (Valeur 2001, Drezek et al. 2001). The bands from 435 and 463 nm (λex = 380 nm) might be produced by substituted β-carboline compounds (Stachel et al. 1999). The higher intensity of the 463 nm band from G sample point to a higher content of β-carboline

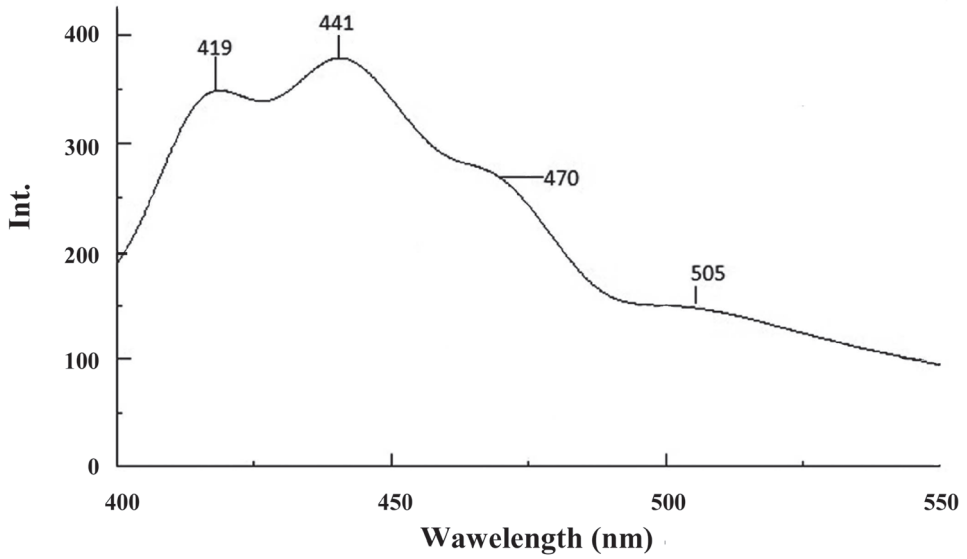

Figure 7. FP spectra of the sample of solid phase of *M. graniger dragani* (λex = 380 nm) (Int.- intensity of the peak).

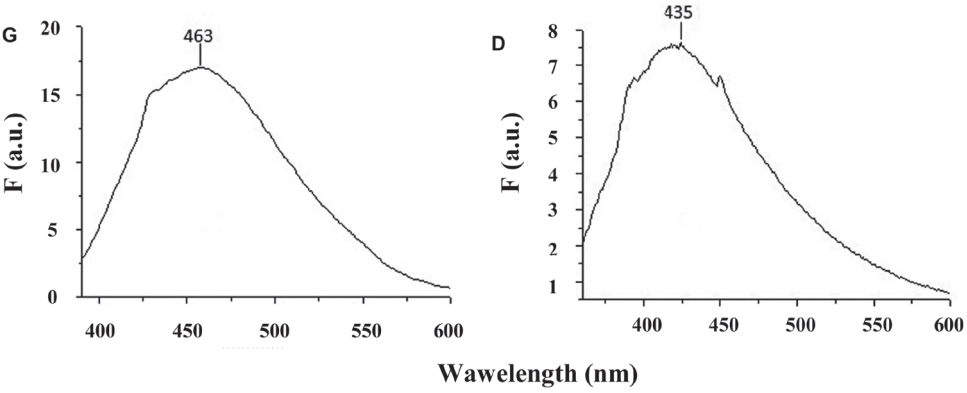

Figure 8. FP spectra of the samples of ethanol extracts of *M. graniger graniger* (**G**) and *M. graniger dragani* (**D**) samples (λex = 380 nm) (F (a.u.) = fluorescence arbitrary units).

derivative, which might be attributed to its formation and accumulation over time in the sample (Fig. 8).

The differences between the emission bands (excitation at 380 nm) of the solid samples and ethanol extracts might be due to the formation of hydrogen bonds with the involvement of the OH groups of the ethanol, emphasizing the influence of the reaction environment, but also its interactions with the chitin and the traces of conjugated lipids. Also, the different solubility in alcohol of the various compounds leads to differences between the emission bands. The molecular fluorescence (FP) tests confirm

the data provided by the autofluorescence microscopy, allowing the identification of the β-carboline (beside other aromatic compounds) as the main source of the fluorescence.

The investigations by fluorescence microscopy and by spectroscopic molecular analysis showed the presence of fluorescence in the 330–385 nm excitation domains due to aromatic structures, most probably belonging to the β-carboline type, and changes in the polyamide structure at ageing, changes recorded in *M. graniger graniger* and *M. graniger dragani*.

The autofluorescence is characteristic for all observed individuals without respect to their geographic origins. It was confirmed in all tested specimens from the entire area inhabited by *M. graniger*, both subspecies showing the same intensity, and it was found also in *M. alpicola* from elsewhere. Furthermore it was recorded in animals observed in caves as well as in individuals kept in laboratory. The individuals stored for long periods in ethanol in collections retain this property. Accidental contamination of *M. graniger* by any fluorescent compounds from the food or by fluorescent microorganisms restricted to certain caves, is challenged by the universal presence of the autofluorescence in all tested populations collected from different caves in various geographic areas.

The very intensively fluorescent structures on the body surface of *M. graniger* seem to roughly correspond to some of the structures we observed previously on the body surface of this species using scanning electron microscopy (Giurginca et al. 2012). The cephalon, pereion, and pleon of this species are covered by a series of tubercles connected by finer surface structures similar to scales (Fig 9). These scales resemble a honeycomb-like net (polygonal structure) and cover almost the entire body surface. Tubercles have a more intense autofluorescence than the net of polygonal scales (Giurginca et al. 2012).

Both the solid and ethanol extract samples contain proteins (polypeptides), chitin (N-acetyl glucosamine) and calcite also identified spectrally in FT-IR and by XRF. It corresponds with general information about the body composition of terrestrial isopods (Wood and Russell 1987, Becker et al. 2005, Giurginca et al. 2010). This composition shows structural changes even when the material is stored in ethanol due to oxidative and enzymatic ageing processes. These aspects of ageing are known for other polypeptide types, for instance for collagen from the human and animal skin, many data coming from studies on new and historical parchments and from leathers tanned with various agents (Badea et al. 2008, Dolgin et al. 2009, Miu et al. 2007). All these changes are described by detailed studies made by IR (MID and NIR), UV-VIS, and FP molecular spectroscopy as well as by other physical and chemical techniques (Badea et al. 2008).

Our observations underline mainly changes of the polypeptides structure by chain alteration (the disappearance of amide III in the case of G sample), crosslinking and the forming of dityrosine and other polycondensated compounds, among which β-carboline due to oxidative processess. We have to stress that β-carboline is present in the body of living animals as a result of their natural ageing and it is not only the result of ageing of material stored in alcohol.

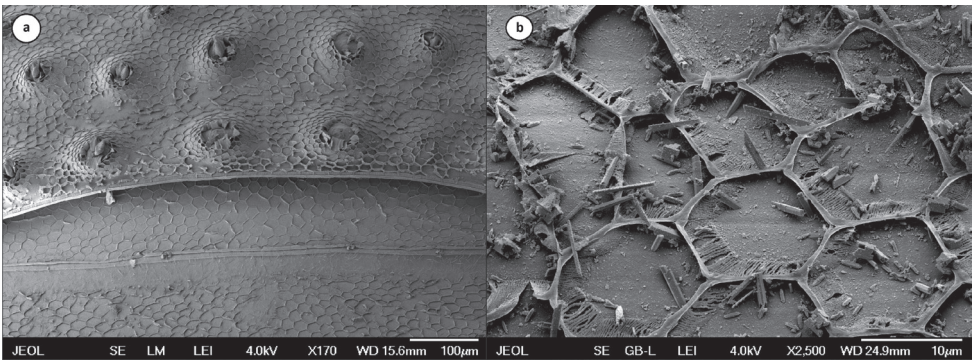

Figure 9. First and second pereionites of *M. g. graniger* showing the position of tubercles (**a**); detail of the honeycomb-like net of scales at *M. g. dragani* (**b**) (after Giurginca et al. 2012 modified).

The microscopically observed blue fluorescence of *M. graniger* as a response to excitation UV light (about 350 nm) corresponds to the wavelengths range of the blue colour (approximately 450–495 nm after Bruno and Svoronos 2005). Spectroscopic parameters of samples preserved in ethanol, indicated that the autofluorescence emitting blue light observed from the living individuals of *M. graniger* may be due to some β-carboline or coumarine derivatives, by some crosslinking structures, dityrosine or due to other compounds showing similar excitation – emission characteristics. The β-carboline or coumarine derivatives were reported to be together responsible for the autofluorescence of scorpions (Stachel et al. 1999, Frost et al. 2001). However, the definitive solution of the problem of the chemical fundament of the autofluorescence of *M. graniger* may bring the isolation and analysis of fluorescent compounds as was performed in scorpions by Frost et al. (2001). In a subsequent study, we will follow a non-spectroscopic analytical approach, such as chromatography and other methods.

The functional advantage of invertebrate fluorescence is not yet known regardless of many hypotheses discussed in literature (see Wankhede 2004 or Gaffin et al. 2012). Some observations (Stachel et al. 1999, Wankhede 2004) suggest that the intensity of scorpions autofluorescence is linked to the sclerotisation of cuticle. It is accepted that chemical linking of cuticular proteins can lead to broad-spectrum fluorescence (Wankhede 2004). The dimerization of the cyclic amino acids, tyrosine and tryptophan, leads to the fluorescent compounds resilin and β-carbolines (Stachel et al. 1999). It is possible that fluorescence is not an adaptive feature but just a side effect of a metabolic product with other functional significance or no functional significance at all, as in waste material.

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Supplementary material 1

Autofluorescence of living *Mesoniscus graniger*

Authors: Andrei Giurginca, Vladimír Šustr, Karel Tajovský, Maria Giurginca, Iulia Matei

Data type: MPEG video file

Explanation note: Living *M. graniger* individuals recorded by Canon EOS camera on the cave sediment inside Ardovská Cave (Slovak Karst, Slovakia) under white LED lamp and UV lamp consecutively.

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RESEARCH ARTICLE

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Demography of some non-native isopods (Crustacea, Isopoda, Oniscidea) in a Mid-Atlantic forest, USA

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Abstract

Introduced species dominate the terrestrial isopod fauna in most inland habitats of North America, including urban landscapes. These non-native species are often very abundant and thus potentially play a significant role in detritus processing. We monitored isopod assemblages in an urban forest for a year to examine the relationship between surface activity and abiotic environmental factors, and to analyze reproductive characteristics that might contribute to their successful establishment. Using pitfall trap samples we recorded five species, two of which, *Trachelipus rathkii* and *Cylisticus convexus*, were highly abundant. We determined size, sex and reproductive state of each individual. Surface activity of both species reflected variability in abiotic stress factors for isopods, such as soil moisture and soil temperature. Early spring the main trigger was soil temperature while later in the season increasing temperature and decreasing soil moisture jointly affected population dynamics. Activity significantly correlated with soil moisture. The temporal pattern of sex ratios supported the secondary sex ratio hypothesis. Males dominated the samples on the onset of the mating season in search of females. The pattern was reversed as females searched for suitable microsites for their offspring. Size independent fecundity decreased as conditions became more stressful late in the season.

Keywords

Abiotic drivers, activity density, reproductive patterns, secondary sex ratio hypothesis, urban soil fauna

Introduction

In recent years there has been an increased interest in non-native, expansive soil invertebrates in North America. Studies almost exclusively focused on earthworm invasion (e.g. James and Hendrix 2004, Hale et al. 2005, Szlavecz et al. 2011a). Earthworms, as ecosystem engineers have multiple, profound and visible effects on soil physical and biogeochemical processes. However, the soil decomposer food web is complex, and other members of the fauna also contribute to these processes. Soil invertebrate community structure with special emphasis of non-native species other than earthworms, has received less attention, partially because their effects on ecosystem processes may be more subtle (Niemelä et al. 1997).

Terrestrial isopods are macro-decomposers that can significantly contribute to detritus processing (comminution, inoculation) and nutrient release. They occur also in habitats too extreme for earthworms, such as salt marshes, arid grasslands and deserts. Here and in other habitats they can reach extremely high local densities (e.g. Paris and Pitelka 1962, Steinberger 1976, Sorensen and Burkett 1977, Shachak et al. 1979, Dias et al. 2005, Messina et al. 2012) elevating them to the rank of the primary detritivore grazers and keystone group in regulating fungal communities (Crowther et al. 2013).

About one-third of the North-American Oniscidea is non-native. The endemic species mostly concentrate in coastal areas, caves, and the southern regions of the continent (Leistikow and Wägele 1999, Jass and Klausmeier 2000). Introduction of non-native woodlice, mostly from Europe, has been going on for centuries. Many of these species are synanthropic, and, probably due to lack of native fauna, successfully invaded wildland habitats, agricultural fields and cities. Isopods are among the most abundant arthropods in urban landscapes (Bolger et al. 2000, Smith et al. 2006, Vilisics et al. 2007a). Undoubtedly, life history characteristics of successful species at least partially explain their dominant status.

In this paper we report data on isopod demography in an urban forest in Baltimore, Maryland, USA. The study was part of a larger ongoing monitoring effort coordinated by the Baltimore Ecosystem Study (www.beslter.org, BES thereafter). BES is one of the two urban sites within the Long Term Ecological Research (LTER) network in the USA. One overarching question BES explores how heterogeneity in social, physical and biological factors interact to influence biodiversity (including soil biodiversity) at multiple scales (Swan et al. 2011, Szlavecz et al. 2011b). In the present study we examined the relationship between isopod population characteristics and abiotic environmental factors, and further analyzed reproductive characteristics that might explain high local abundance and thus invasion success of the dominant species.

Methods

Study site

We surveyed the isopod fauna in Leakin Park, a 492 ha contiguous parkland in Baltimore, Maryland, USA (39°15'N, 76°30'W). The park is about 8 km NW from the urban core,

heavily forested, and surrounded with high density residential areas. The 90 year old forest belongs to the Tulip poplar Association (Brush et al. 1980) with common canopy species including Tulip poplar (*Liriodendron tulipifera*), several oaks (*Quercus alba*, *Q. coccinea*, *Q. velutina*), and American beech (*Fagus grandifolia*). Oaks and Tulip poplar make up 77% of the total annual litter mass, which is 4122 kg ha^{-1} (Groffman et al. 2006). The soil belongs to the Legore series (fine-loamy, mixed, mesic, Ultic Hapludalf). Duff layer is thin (0–2 cm), bulk density and pH of A horizon are 1.12g cm^{-3}, and 5.1, respectively. More detailed description of the vegetation and soils is given in Groffman et al. (2006).

The climate can be characterized by hot humid summers and cold winters with average annual air temperatures ranging from 14.5 °C in the inner urban areas to 12.8 °C in the surrounding rural areas. Precipitation is distributed evenly throughout the year in the region and ranges from an annual average of 106.8 cm in Baltimore to 103.1 cm in the surrounding metropolitan area (NOAA, www.nws.noaa.gov).

Sampling and laboratory measurements

Terrestrial isopods were sampled using pitfall traps (250 ml plastic cups) filled with propylene glycol. Ten traps were placed randomly around a 40 m × 40 m permanent forest plot established by the Baltimore Ecosystem Study LTER (Groffman et al. 2006). Traps operated between October 1999 and November 2000 and were emptied monthly except in late fall-winter when surface activity is generally low. The material was stored in 70% ethanol. All individuals were identified to species level using the nomenclature by Schmalfuss (2003).

Population and reproductive characteristics were determined only for the two abundant species. Because pitfall trap samples indicate a combination of surface activity and abundance of epigeic invertebrates, obtaining even relative density information creates a challenge. Recently, the term 'activity-density' has been used (Melbourne 1999, Westerman et al. 2008) to express abundance, and here we follow this practice. Activity density is expressed as number of individuals caught per trap per day.

We estimated body size by measuring the widest point of the head capsule (cephalon) at the level of the eyes (Sutton 1968). Measurements were taken to 0.01 mm accuracy under a dissecting stereo microscope. Adult females were divided into three reproductive categories: non-reproducing, gravid (either with eggs, embryos or mancas), and post-reproductive (with empty marsupium). Reproductive period was defined as the time span between the appearance of the first gravid females and that of the last one with brood pouch (marsupium) either with or without progeny (Sutton et al. 1984).

Abiotic factors

We obtained soil temperature and moisture data from the Baltimore Ecosystem Study database. Soil temperature was measured continuously using HOBO H8 Pro Series

Temp/External Temp data loggers at 10 cm depth. For soil moisture measurements six time domain reflectometry (TDR) waveguide probes (Soil Moisture Equipment Corporation) were installed vertically into the soil at random locations throughout the plot. The waveguide probes are 20 cm long, so those vertically installed span a depth of 0 to 20 cm below ground. Soil moisture was measured once every four to six weeks.

Statistical analysis

Mean daily values were used to explore correlation between soil temperature and activity density. Distance-weighted least squares fitting was used for smoothing. Due to lack of continuous soil moisture data, relationships between activity density and soil moisture was explored by using Spearman Rank Order Correlation Coefficient. We used multiple linear regression to explore relationships among fecundity (number of eggs produced by females), body size (cephalon width) and sampling date for each species. For computing these analyses we used STATISTICA 12 software (StatSoft Inc. 1984–2013).

To compare relative importance of independent variables i.e. size and time, we used standardized beta partial regression coefficients. Beta coefficients are obtained by setting all the variables to a mean of 0 and standard deviation to 1.

Ratio of males is expressed as the total number of males (M) over total number of adults (N) caught during a given trapping period (Dangerfield and Telford 1994). We conducted two different statistical tests. First, we wanted to estimate the unknown sex ratio and its uncertainty. Second, we wanted to assess the probability of the null hypothesis that the observed data is consistent with a M:N ratio of 0.5. A simple chi-square test informs only of the latter, moreover, chi-square test assumes Gaussian distribution i.e. symmetric error bars. This approximation is only valid when N is large. Since several of our samples were small, we chose a different approach (Gotelli and Ellison 2004). At a given sex ratio the distribution of observed M males out of a sample of N individuals (M<N) follows a binomial distribution. We first calculated the cumulative probability (CDF) that the number of males in the sample is equal or less than the measured count M. If M is more than half the sample size we estimate the probability that the number of males is equal or more than the count. Using this cumulative binomial distribution, we first computed the 95% confidence intervals in both directions, which often are not symmetric (Table 1). Finally we determined the probability the observed number of males is consistent with a sex ratio of 0.5, by simply reading off the midpoint value of the CDF.

Results

During the course of sixteen months a total of 2480 isopods were caught. The following five species were recorded: *Haplophthalmus danicus* Budde-Lund, 1880, *Hyloniscus riparius* (C. Koch, 1838), *Philoscia muscorum* (Scopoli, 1763), *Trachelipus rathkii* (Brandt, 1833) and *Cylisticus convexus* (De Geer, 1778). The pitfall material was domi-

Table 1. Abundance of *Trachelipus rathkii* and *Cylisticus convexus* between October 1999 and November 2000 in Leakin Park urban forest, Baltimore, USA. Numbers from all pitfall traps are pooled for each sampling period. Male ratio was calculated as proportion of males in total sample. 95% confidence intervals for these estimates are given the parentheses. Bold letters indicate significant differences from the expected 0.5 value.

Species	Month	Males	Females	Ratio of males	*p*
Trachelipus rathkii	October	110	118	0.48 (0.43; 0.54)	0.321
	November	38	61	**0.38** (0.31; 0.47)	**0.013**
	January	2	2	0.50 (0.25; 0.91)	0.312
	March	9	25	**0.26** (0.17; 0.42)	**0.004**
	April	76	58	**0.57** (0.50; 0.64)	**0.050**
	May	462	445	0.51 (0.49; 0.54)	0.275
	June	244	306	**0.44** (0.41; 0.48)	**0.005**
	July	401	461	**0.47** (0.44; 0.50)	**0.022**
	August	141	230	**0.38** (0.35; 0.43)	**< 0.001**
	September	90	128	**0.41** (0.37; 0.48)	**0.006**
	October	16	21	0.43 (0.32; 0.58)	0.256
	November	2	4	0.33 (0.15; 0.73)	0.343
Cylisticus convexus	October	185	252	**0.42** (0.39; 0.47)	**0.001**
	November	38	55	**0.41** (0.34; 0.50)	**0.048**
	January	0	0	NA	NA
	March	4	8	0.33 (0.18; 0.62)	0.194
	April	11	20	0.35 (0.24; 0.52)	0.075
	May	178	315	**0.36** (0.33; 0.40)	**< 0.001**
	June	263	353	0.51 (0.48; 0.55)	0.298
	July	352	568	**0.38** (0.36; 0.41)	**< 0.001**
	August	353	163	**0.61** (0.57; 0.65)	**< 0.001**
	September	162	227	**0.42** (0.38; 0.46)	**0.001**
	October	48	38	0.56 (0.47; 0.65)	0.117
	November	6	16	**0.27** (0.16; 0.47)	**0.026**

nated by the latter two species, with 1270 *T. rathkii* and 1073 *C. convexus* (53 and 45 %) individuals, respectively. Detailed analysis of the population characteristics is given only for these two species.

Surface activity, demographic changes

Isopod numbers began to increase late April, peaked in July and declined in September and ceased by November (Fig. 1A and B). Activity density of both species positively correlated with soil temperature (r = 0.75, and r = 0.87, for *T. rathkii*, and for *C. con-*

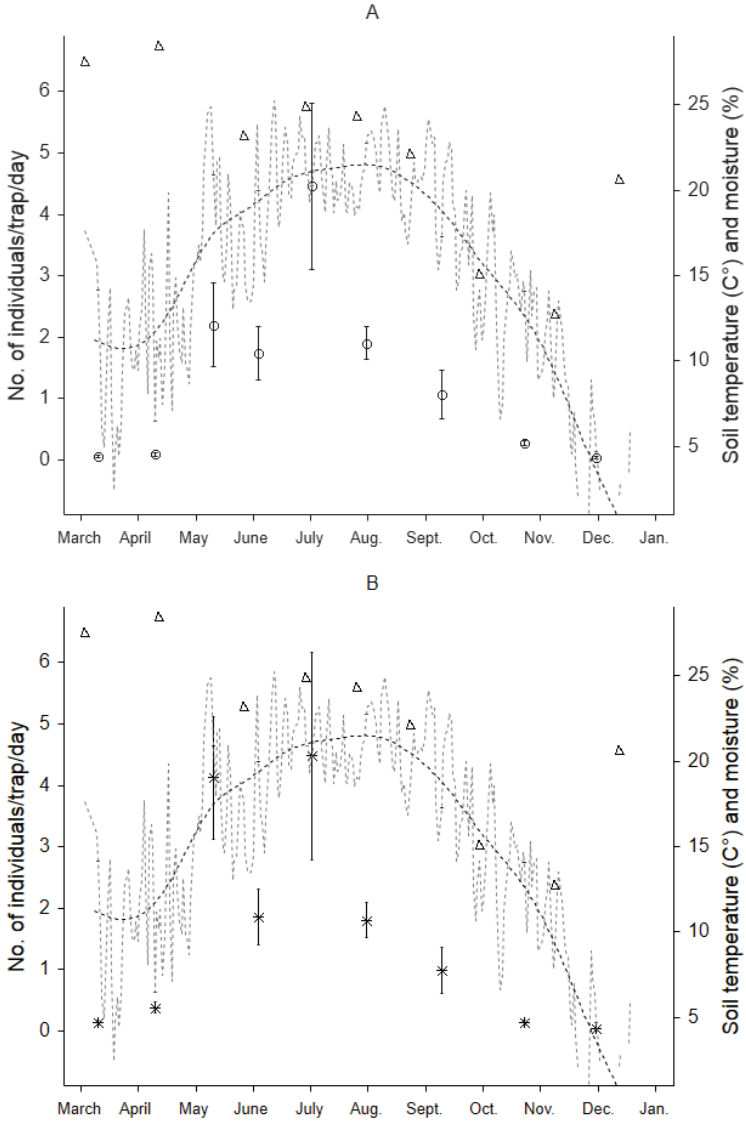

Figure 1. Temporal changes in activity-density of *Cylisticus convexus* (**A**) and *Trachelipus rathkii* (**B**) and soil physical characteristics in an urban forest in Baltimore. Mean numbers of individuals captured daily per trap (*C. convexus*: open circles, *T. rathkii*: asterix) ± SE are shown. Dotted line: mean daily soil temperature at 10 cm; dashed line: smoothed soil temperature; open triangles: volumetric soil moisture content.

vexus, respectively). Soil moisture decreased during the growing season (Fig. 1A, B). Excluding winter months, when no isopods were caught, isopod activity positively correlated with soil moisture during the growing season (Spearman R = 0.32, $p < 0.05$ for *T. rathkii*, and R = 0.35, $p < 0.05$ for *C. convexus*).

Reproductive characters

Male ratio

Male ratio varied over time, with the highest and lowest male : total ratio being 0.57 and 0.26 for *T. rathkii*, 0.61 and 0.27 for *C. convexus*, respectively (Table 1). For *T. rathkii*, we detected significant deviations from the expected the 0.5 ratio in seven sampling dates. In all but two months (April, May) activity density of females exceeded that of males. For *C. convexus* we detected significant differences also in seven cases. Again, only two months did males exceed females, but this happened later in the season (August, October).

Reproductive period, phenology and fecundity

Trachelipus rathkii started reproducing late April – early May (Table 2) with 20% of the females being gravid, and all in the egg carrying stage. Proportion of gravid females peaked at 57% in June–mid-July and slightly declined (47%) by mid-August. Gravid *C. convexus* females appeared in the traps later in the season (June–July), but at this period females both with eggs and empty marsupium were present. In September only a single gravid *T. rathkii* was caught, while 16% of the 146 female *C. convexus* in the sample were still gravid, all in post-reproductive stage.

Fecundity of females in *C. convexus* and *T. rathkii* were compared over time. We analyzed the relationship between fecundity and body size using multiple linear regression models: clutch size (number of eggs), as dependent variable, and body size (head width) and time (days from the start of the investigation), as independent variables. Regression summary for *C. convexus*: adjusted $R^2 = 0.65$, $F(2,119) = 112.10$, $p < 0.001$; beta(day) = -0.44; beta(head width) = 0.37. Regression summary for *T. rathkii*: adjusted $R^2 = 0.38$, $F(2,123) = 38.836$, $p < 0.001$; beta(day) = -0.22; beta(head width) = 0.65. Number of eggs increased with body size (Figure 2A), and these relationships were sta-

Table 2. Percentage of reproductive *Trachelipus rathkii* and *Cylisticus convexus* in Leakin Park, Baltimore. Two stages are distinguished. Gravid: with eggs, embryos or mancas in the marsupium; postreproductive: empty marsupium.

			May	June	July	Aug
Trachelipus rathkii	All reproductive*		20.6	57.1	47.1	0.6
		Gravid**	100	41.7	17.5	0
		Postreproductive**	0	58.3	82.5	100
Cylisticus convexus	All reproductive*		0	61.0	40.0	16.0
		Gravid**	0	29.4	57.1	0
		Postreproductive**	0	70.6	42.9	100

*Calculated as percentage of all females in the sample

** Calculated as percentage of all reproductive females in the sample

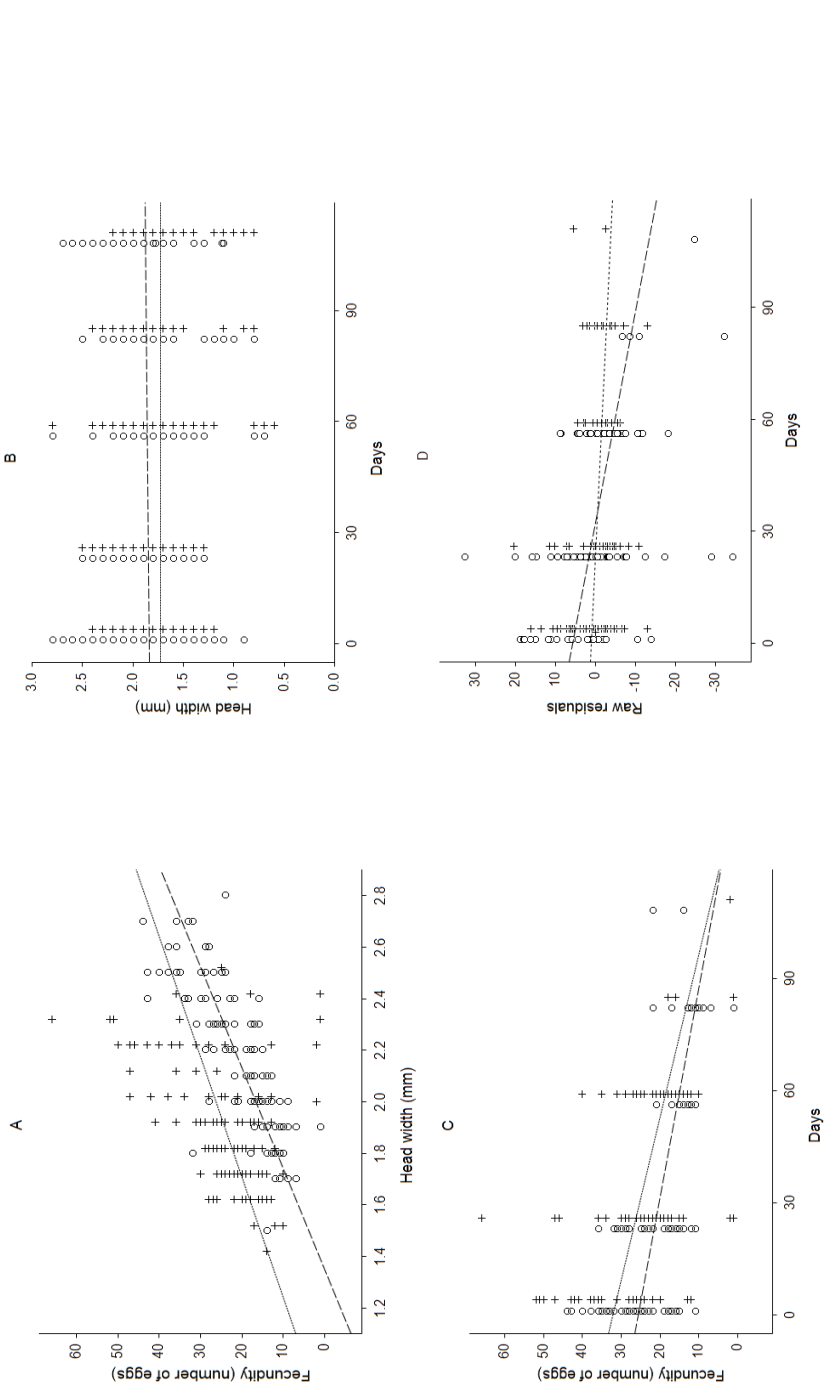

Figure 2. Fecundity of the dominant isopod species in Leakin Park, Baltimore. **A–B**: Relationship between fecundity and size (**A**) and its stability over time (**B**) **C–D**: Change of fecundity over time (**C**) and size independent fecundity over time, based on the residuals of egg numbers (**D**). *Cylisticus convexus*: open circles and dashed lines; *Trachelipus rathkii*: crosses and dotted lines.

ble throughout the season (Figure 2B). Size independent fecundity decreased with time for both species (Figure 2C): residuals of egg numbers showed negative trends, however, their slopes were different (Figure 2D). Standardized beta partial regression coefficients were -0.44 ($p < 0.001$) and -0.22 for *C. convexus* and *T. rathkii*, respectively, indicating that the decrease of female fecundity during the season was not linked to body size.

Discussion

Species composition

In the Greater Baltimore Metropolitan Area we have recorded a total of eleven terrestrial isopod species (Hornung and Szlavecz 2003). All species are non-native, and most have been known from North America for a hundred years (Hatch 1947, van Name 1936, 1940, 1942, Eberly 1953, Lindroth 1957, Jass and Klausmeyer 2000). Urban fauna is often characterized by synanthropic, generalist species that may lead to higher community similarity among cities (McKinney 2006). All five species present in Leakin Park fit this category, and the two dominant species *Trachelipus rathkii* and *Cylisticus convexus*, are among the ten most abundant exotics in North America (Jass and Klausmeier 2000). Both species are characterized as expansive in Europe and have been introduced to other continents, as well (Gruner 1966). In Europe both species occur in a variety of habitats, including cities (Korsós et al. 2002; Hornung et al. 2007, 2008, Vilisics and Hornung 2009, Vilisics et al. 2007a,b). The difference between their occurences in the two continents is that *C. convexus* tends to be more synanthropic in Central and Western Europe, its abundance is moderate to low, and is scarce or missing in the North (Gruner 1966, Berg et al. 2008, Vilisics et al. 2007a). In North America both species are widely distributed, and abundant populations of *C. convexus* and *T. rathkii* were reported e.g. from Michigan (Hatchett 1947) and Wisconsin (Jass and Klausmeier 1996), too.

Temporal patterns

Temperature and relative humidity are known to be the main drivers of terrestrial isopod activity (Warburg 1987, 1993). Our data support this statement, but also show a more complex relationship in the field. Soil temperature is the main factor triggering surface activity early spring; however, later in the season increasing temperature and decreasing soil moisture jointly affect dynamics of the populations. In early fall even though temperature remains high, activity decreases (Fig. 1). At this time soil moisture is low due to high evapotranspiration rates by trees and lack of precipitation (Groffman et al. 2006). We acknowledge that other factors, such as size, mobility, and behavior may bias pitfall trap samples. However, the huge number of individuals in the samples gives us confidence that the dynamics we detected for these two abundant species, is real.

Reproductive characteristics

Male ratio

With the exception of parthenogenetic species, where males occur in extremely low numbers, the sex ratio of most isopod species can be described by bimodality (e.g. Paris and Pitelka 1962, Sorensen and Burkett 1977, Hornung 1989, 1991). There are some examples for species with very different and constant adult sex ratio, e.g. *Porcellio ficulneus* Budde-Lund, 1885 male: female = 1:9 (Warburg 2007), and *Schizidium tiberianum* Verhoeff, 1923, 1:6 (Warburg and Cohen 1991). Sex ratio for terrestrial woodlice is routinely reported in papers focusing on population characteristics. In most cases these values are only snapshots obtained from samples of a short time period and do not reflect temporal changes of the sex ratio. Sex ratio might differ seasonally depending on different mortality and/or activity of sexes. We are aware of only a few long term studies where populations were frequently sampled to obtain changes of sex ratio over time (e.g Paris and Pitelka 1962, Sorensen and Burkett 1977). It is therefore important to excercise caution when comparing data for different populations even for the same species, as variations both in space and time may occur. Montesanto et al. (2008) reported a special case for *Platyarthrus aiasensis* Legrand, 1954 comparing 19 populations in and around Sicily. The populations differed in male ratio from 0 (parthenogenetic) to 0.37 of males.

Deviation from the expected 0.5:0.5 ratio may be due to behavioral differences between the sexes especially during reproductive period as proposed by the secondary sex ratio hypothesis (Dangerfield and Hassall 1994). According to this hypothesis at the onset of the mating season males are more active looking for receptive females. Later, gravid females exceed males in the sample, as they are looking for favorable microhabitats that maintain optimal conditions for their progeny. Both *T. rathkii* and *C. convexus* follow this pattern in our study site; the difference between them is the peak of timing of male searching behavior. High male dominance (0.98) was found also for *Protracheoniscus politus* (C. Koch, 1841) in Hungary (Oberfrank et al. 2011) and for *Armadillidium vulgare* (Latreille, 1804) in Texas (Sorensen and Burkett 1977) at the beginning of the activity season, before the onset of reproduction.

Gruner (1966) compiled and qualitatively reported population data for the two species we studied here. The percentage of *C. convexus* males was found to be lower than 50% in Denmark, France and Italy. For *T. rathkii* a female predominance was also reported.

Reproductive period and phenology

In Europe the onset of the reproductive period varied with latitude for both *C. convexus* and *T. rathkii* (Gruner 1966). For instance, *C. convexus* started reproducing in April in France; in Denmark the onset shifted to June. The number of offspring ranged between

14–50 per female, with extremely high numbers (73 embryos per female) in Italy (Gruner 1966). *T. rathkii* was reported to have two broods between May and September.

Jass and Klausmeier (2004) found a latitudinal difference in reproductive peaks in North America for several species including the ones studied here. In Wisconsin gravid females of *C. convexus* were present during June-July in the populations while reproductive period of *T. rathkii* was longer (June-August). In the present study the reproductive period started earlier, in April both for *T. rathkii*, and for *C. convexus*, and ended in August and September, respectively.

Reproductive output over time

Reproductive output is an important component of life history strategies and has a cost of decreased parental survival (Stearns 1976). The strong correlation between female size and egg number in terrestrial isopods is well known (e.g. Dangerfield and Telford 1990, Ma et al. 1991, Nair 1978, Warburg 2011). However, females may not allocate the same amount of energy into reproduction during the entire season. We found that the mean number of eggs/embryos decreased independently of female size as the reproductive period progressed. This tendency is the consequence of increasing environmental stress (unfavorable humidity and temperature changes, drought) and contributes to the costs of survival (Cody 1966, Charnov 2002, Hornung and Warburg 1993, 1994, 1998). Isopods can either invest less or regain part of the energy invested in reproduction earlier by reabsorbing ovarian oocytes or marsupial eggs under stressful conditions (Hornung and Warburg 1993, 1994).

Successful establishment, expansion, invasion

There is still some confusion regarding terminology in invasion ecology. The term invasive species is used for species "exhibiting rapid spread, irrespective to impact" (Davis 2009), as well as for species showing "demonstrable ecological or economic impact" (Lockwood et al. 2007). The two isopod species we reported here undoubtedly fit the first description, as both are well established and common throughout North America (Jass and Klausmeier 2000). The main characteristics successful invaders share include high dispersal rate, high genetic variability, short generation time, large number of offspring, broad diet and ecological tolerance. Some of these traits overlap with what Sutton et al. (1984) describes for "eurodynamic", essentially *r* strategist species. Detritivores are by definition resource generalists (even though they have food preferences) allowing them to find food in a variety of habitats. Both *C. convexus* and *T. rathkii* have high fecundity, and can reproduce several times per season. Moreover, female isopods have been shown to be able to store sperm and utilize their stock in repeated reproduction events (Suzuki and Ziegler 2005). This can be especially significant when only one or a few females are transported from one habitat to another. Isopods are relatively easy

to transport with soil, mulch, ornamental plants, timber and other means. Once introduced, they can quickly establish and, being mobile epigeic species, further disperse on their own. Garthwaite et al. (1995) estimated that *Armadillidium vulgare*, another common species in North America spread across the continent and reached the West Coast in about 150 years. We can only speculate that the lack of native relatives or ecologically equivalent soil fauna further facilitated the spread of terrestrial isopods including the species studied here.

The highly altered urban habitats can serve both as points of introduction via trade or transportation, and refuges for non-indigenous soil fauna. Residential areas provide food (e.g. compost, mulch) and shelter (building foundation, landscaping objects), while green corridors or even underground pipe systems can be conduits for dispersal. High epigeic isopod abundance has been repeatedly shown in urban habitat fragments and suburbs (Bolger et al. 2000, Smith et al. 2006, Vilisics et al. 2007a). Often, the same species dominate urban isopod fauna. *T. rathkii* and *C. convexus* belong to this successful cosmopolitan group, contributing to the global phenomenon of biotic homogenization in cities (Perrings et al. 2010).

Conclusions

We examined population dynamics and reproductive characteristics of terrestrial isopods in an urban forest in Baltimore, Maryland, USA. Temporal patterns of male ratio supported the secondary sex ratio hypothesis for both dominate species. As expected, fecundity was correlated with female size. However, size independent reproductive output declined during the active season indicating a response to increasing stress. High fecundity, good dispersal ability and broad habitat and resource tolerance all may contribute to the invasion success of the investigated species in North America. Additionally, lack of native competitors and locally favorable conditions can further facilitate their spread and persistence in many ecosystems.

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EH processed the samples in the laboratory, identified and measured the isopods, and determined their reproductive state. MD carried out the statistical tests. KS did all field work and sorted the pitfall trap samples. All three authors contributed to writing the manuscript.

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RESEARCH ARTICLE

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Effects of microclimate on behavioural and life history traits of terrestrial isopods: implications for responses to climate change

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Abstract

The sensitivity of terrestrial isopods to changes in both temperature and moisture make them suitable models for examining possible responses of arthropod macro-decomposers to predicted climate change. Effects of changes in both temperature and relative humidity on aggregation, growth and survivorship of species of isopods contrasting in their morphological and physiological adaptations to moisture stress have been investigated in laboratory microcosms.

All three traits were more sensitive to a reduction in relative humidity of 20–25% than they were to an increase in temperature of 5–6 °C. These results suggest that predicted changes in climate in south east England may reduce the extent to which soil animals stimulate microbial activity and hence carbon dioxide (CO_2) emissions from soils in the future. This may help to mitigate the potential for a positive feedback between increased CO_2 emissions from soils, and increased greenhouse effects causing an increase in soil temperatures.

Keywords

Temperature, moisture, aggregation, growth rates, mortality rates, stimulation of micro-organisms

Introduction

Climate change is the greatest human induced environmental challenge ever faced by mankind (Doney et al. 2012, Middleton 2008). In South East England global climate change models predict that by 2060 air temperatures will have risen by 1.4–5.8 °C (Bale et al. 2002), summer rainfall decreased by 50% (Murphy et al. 2009) and be restricted to fewer more intense episodes with more and longer periods of drought (Rowell 2005). Such changes could lead to a reduction in relative humidity at the soil/litter interface (Edwards 2013). One potential consequence of climate change, particularly at higher latitudes, is development of a positive feedback loop, in which warmer temperatures lead to accelerated mineralisation of soil organic matter, therefore to increased emissions of carbon dioxide (CO_2) and methane, leading to an increased greenhouse effect resulting in further increases in soil temperatures (Heimann and Reichstein 2008). Changes in microclimate may affect rates of CO_2 emissions from soils in two ways: directly influencing microbial metabolism and indirectly by influencing the externt to which soil animals regulate microbial metabolic processes (Bardgett et al. 2008, Dias et al. 2012).

Soils contain the world's largest terrestrial stores of carbon, releasing ten times more CO_2 than all anthropogenic emissions combined (Schlesinger and Andrews 2000). It follows that a 1% increase in CO_2 emitted from the soil is equivalent to a 10% increase in anthropogenic emissions (Dias et al. 2012). The majority of CO_2 emitted from the soil is due to microbial catabolism of soil carbon pools (Lavelle 2002), but this process is influenced by soil animals as key system regulators of this process (Coleman et al. 2004). This regulation is achieved by soil animals stimulating microbial activity partly because they redistribute propagules to fresh substrate (Lavelle and Spain 2001) and partly because they can change the substrate physically, including increasing its surface area as a result of comminution (Hassall and Sutton 1977), chemically e.g. by altering pH and nitrogen (McBrayer 1973) and biologically by changing the densities and hence taxonomic composition of microbial populations and communities (Zimmer and Topp 1999).

Members of the soil macrofauna may respond to future climate conditions by both "functional responses" and "numerical reponses", in this paper used in a broad sense of changes in their activities which may alter their "function" in the ecosystem. Specifically we use aggregation as an example of a functional response. Secondly by "numerical responses" which are those responses of life history and population parameters that might influence the density and population dynamics of individual species.

Terrestrial isopods form a dominant component of the soil macrofauna in many ecosystems (Hassall et al. 1987). They are found in a wide range of ecosystems, from xeric deserts (Warburg 1965) to temperate littoral zones (Oliver and Meechan 1993, Paoletti and Hassall 1999). Terrestrial isopods are strongly sensitive to microclimatic conditions and have developed morphological and behavioural traits, such as pleopodal lungs and aggregation, as adaptations to the terrestrial environments (Warburg 1968). The extent to which morphological traits have developed to reduce moisture loss varies between different families: for example members of the Oniscidae lack well developed pleopodal lungs in contrast to members of the Porcellionidae which do have elaborately

developed pleopodal lungs with a large number of fine branched tubules, resulting in a greater surface area for absorbing oxygen with reduced loss of water vapour (Wright and Ting 2006). *Oniscus asellus* was chosen as a representative of the Oniscidae family while *Porcellio scaber* and *Porcellio dilatatus* were used, according to availability, as representatives of the Porcellionidae, both species having elaborate pleopodal lungs.

Aggregation is a behavioural adaption to avoid desiccation, where individuals group together to reduce water loss by creating a shell of higher relative humidy around the aggregate (Allee 1926, Friedlander 1964, Morris 1999, Broly et al. 2013). An isolated individual isopod can lose water three times as fast as one in an aggregation (Brockett and Hassall 2005). However, there is a cost to aggregation, as isopods do not feed while aggregating to shelter (Dias et al. 2012). This can potentially alter the extent to which they stimulate microorganism mediated mineralization of carbon based substrates (Hassall et al. 2010, Dias et al. 2012).

Microclimatic variables also strongly influence the numerical responses of isopods by affecting population characteristics such as, growth rate and survivorship (Edney 1954, Rushton and Hassall 1987). In this paper we present the effects of altering both temperature and relative humidity on species of isopods contrasting in both their morphological and aggregative responses to changes in microclimate.

We test the following hypotheses:

1 As relative humidity decreases aggregation and mortality rates will increase while growth rates will decrease.
2 As temperature increases aggregation, growth rates and mortality rates will all increase.

Materials and methods

Experimental design

In the experiments on responses of aggregation behaviour to different temperatures and relative humidities five treatments were used: for responses to temperature 14, 17, 19, 21 or 23 °C and for responses to relative humidity 50, 60, 70, 80 or 90%. These ranges were chosen to bracket the range of microclimate conditions that might be encountered at the litter/soil interface of soils in south east England. 58 replicate arenas were used for each set of microclimate conditions. In both experiments *Oniscus asellus* was used as a representative of the Oniscidae and *Porcellio scaber* as a representive of the Porcellionidae for investigating responses of aggregation behaviour to temperature and *Porcellio dilatatus* as a representative of the Porcellionidae for investigating responses of aggregation to relative humidity.

O. asellus and *P. dilatatus* were also used to investigate responses of relative growth rate and mortality. A 2 × 2 factorial experimental design was used with temperatures of

18.5 °C and 13.5 °C and relative humidities of 90% and 70%. Each treatment combination was replicated 10 times. Differences in temperature of 5 °C, represent close to the higher predicted increases in air temperatures during the 21st century (Bale et al. 2002). As predictions of how climate change might influence relative humidity at the soil/litter interface are not yet readily available, a 20% decrease in relative humidity was chosen as being the range observed within spring and summer in the litter layer of a fixed dune grassland (Hassall 1976).

Experimental protocols

Responses in aggregating behaviour

For investigating responses in aggregating behaviour to temperatures, base cultures of *Oniscus asellus* and *Porcellio scaber* were kept at 23 °C in the dark in 60 × 40 × 16 cm plastic containers with sloping bases of moist plaster of Paris from 0.5–4.0 cm deep covered with 6–2 cm sand to give a level surface with a moisture gradient along the length of the box and with pieces of bark for shelter and a mixture of leaves from broad leaved trees scattered over the sand surface for food. For investigating responses of aggregation to different relative humidities *Oniscus asellus* and *Porcellio dilatatus* base cultures were kept at 23 °C in the dark in 28 × 9 × 15.5 cm plastic containers with a base of 2 cm dampened plaster of Paris covered by 3cm sand and leaf litter. A mixture of broad leaved tree species provided food and cover. The boxes were sprayed with water at approximately 2 day intervals. For both experiments a soft paintbrush and a plastic weighing boat were used to transfer 10 individuals of a single species into 90 mm diameter Petri dishes divided into eight equal sections. Petri dish lids were replaced by a piece of nylon mesh (Hassall et al. 2010).

Experimental arenas were transferred to and from the middle shelf of SANYO versatile environmental test chambers set to 90% RH for investigating responses to temperature and at 22 °C for investigating responses to relative humidity. Arenas were left for 20 minutes to allow arenas to equilibriate to experimental temperatures. They were then removed, photographed and the number of individuals in each section were counted. If an individual was on the dividing line between two segments it was recorded as in the section in which the largest percentage of its body was situated. If the individual was exactly half way over the boundary then it was recorded as being in the section in which the head end was situated (Hassall et al. 2010). After recording their dispersion in the arena, the isopods were returned to the holding container and not used again that day. The Petri dishes were wiped with 70% ethanol to remove aggregation pheromones (Devignes et al. 2011) and were not used again that day.

The variance mean: ratio for numbers in segments was used as an index of aggregation. Aggregation indices were analysed in SPSS, using a two-way ANOVA, and Tukey *post hoc* comparison of means to compare species and humidities.

Responses of growth and mortality rates

Three males and three females of both *Oniscus asellus* and *Porcellio dilatatus*, were divided into size classes and placed into a plastic container measuring 28 × 9 × 15.5 cm (Hassall et al. 2003) lined with 2 to 3 cm of plaster of Paris covered by a 1.5 to 2 cm deep layer of damp sand (Helden and Hassall 1998). Each container had a circular 60 mm diameter wooden shelter supported by a cork above a 38 mm × 10 mm deep plaster of Paris filled Petri dish as a base and a 40 mm Petri dish lid embedded in the sand at the opposite end of the arena as a food container.

Containers were kept in SANYO versatile environmental test chambers and sprayed daily with the average mean summer rainfall of 1.65 mm (Moss and Hassall unpublished). The containers were kept in darkness for the full four weeks (Rushton and Hassall 1987). Isopods were removed from the containers and weighed individually on a Mettler B204 balance every week for four weeks. The population density was sustained throughout the four weeks by replacing any animals that died. The growth rates of the replacements were not monitored as they had not been under the experimental conditions for the same length of time as the original experimental individuals (Hassall et al. 2003).

The growth rate was calculated using relative growth rate, RGR (Helden and Hassall 1998):

$$RGR = [\log(LW_{t1}) - \log(LW_{t0})] / T$$

where LW_{t0} is the live mass (mg) at the start of the experiment, LW_{t1} is the live mass four weeks later at the end of the experiment and T is the number of days from the start to the end of the experiment. The number of isopods of each species that died in each container was recorded weekly. Average values for growth rates in each container were used in the analyses to avoid pseudoreplication. The data were analysed using hree-way ANOVA in SPSS following testing for normality using the Kolmogorov-Smirnov test. *Post hoc* Tukey tests were used to compare different means. Mortality rates per week were analysed using the Mann Whitney U test as the data were not normally distributed.

Results

Responses in aggregating behaviour

Differences in aggregation under different microclimate conditions for both *Oniscus asellus* and *Porcellio scaber* varied significantly with temperature. For both species the most significant increase occurred between 14 and 17 °C (Fig. 1a, b). For *P. scaber* aggregation peaked at 19 °C and then decreased as the experimental animals increasingly

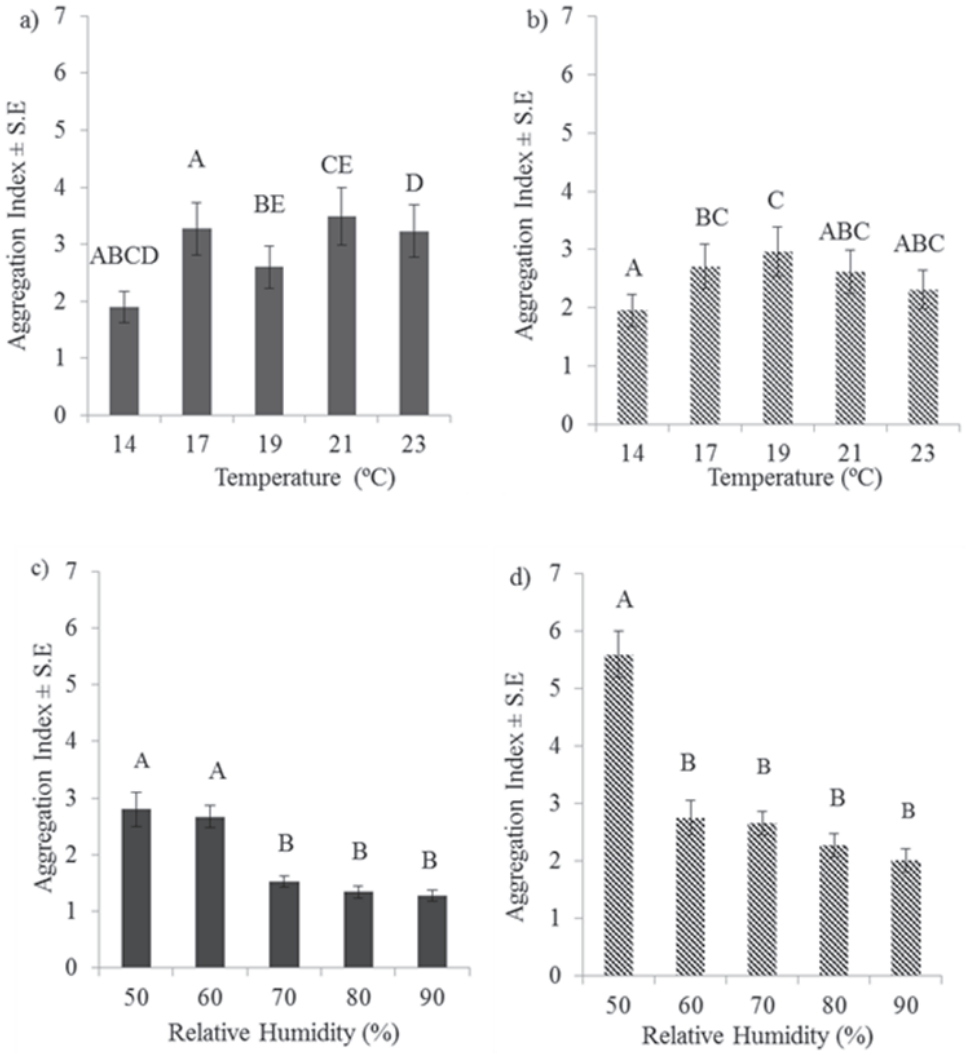

Figure 1. Responses in aggregation index to differences in temperatures and relative humidity: Responses to different temperatures by a) *Oniscus asellus*, ($F_{4, 249}$ = 12.22; P < 0.001) and b) by *Porcellio scaber* ($F_{4,249}$ = 3.76; P < 0.001). and to different relative humidies by c) *Oniscus asellus*, ($F_{4, 230}$ = 25.39; P < 0.001) and d) by *Porcellio dilatatus* ($F_{4,171}$ = 16.85; P < 0.001). Means sharing the same letter are not significantly different from each other at P < 0.05.

switched from aggregating to escape behaviour: walking rapidly round the arena and climbing the walls.

In order to summarise results those for 17–23 °C were combined (average temperature 20 °C) for comparison with the significantly lower results for 14 °C. This showed that for *O. asellus* aggregation was 64% higher and for *P. scaber* 28% higher, following a 6 °C increase in temperature. Aggregation also changed significantly (P < 0.001) for

both *O. asellus* and *P. dilatatus* at different relative humidities (Fig. 1c, d). For *Oniscus asellus* the significant change was between 60% and 70% relative humidity (Fig. 1c). For *P. dilatatus* significant change occurred between 50% and 60% relative humidity (Fig. 1d). Summarising the results by averaging values for treatments that did not differ significantly, showed that there was an increase of 128% in the aggregation of *O. asellus* and 99% increase for *P. dilatatus* when relative humidity decreased by, on average, 25%.

Aggregation of *O. asellus* increased by more than double the increase in aggregation shown by *P. scaber* for the same change in temperature. *O. asellus* also responded more to changes in relative humidity than *P. scaber*. Averaging the results for the two species by combining results for the two temperatures showed that for both species combined aggregation increased by 114% for a decrease of 25% in relative humidity (average value for F = 21.1) compared with a 46% increase in aggregation for a 6 °C rise in temperature (average value for F = 8.0).

Responses in growth and mortality rates to different microclimatic conditions

Results of the 2 × 2 factorial experiment to investigate responses of growth and mortality to a 5 °C rise in temperature and a 20% reduction in relative humidity for *O. asellus* and *P. dilatatus* are shown in Fig. 2a–d. For only one of the four species/relative humidity combinations was there a significant increase in growth rate at the higher temperatures (at 70% RH comparing 13.5 and 18.5 °C for *P. dilatatus* (Fig. 2b)) In contrast for both species at both temperatures, growth rates were significantly higher at 90% RH than at 70% RH. These results thus indicate a more consistent decrease in growth rates for a 20% decrease in relative humidity than increases in growth rates for a 5 °C increase in temperature.

Similarly for mortality for one of the four combinations of species and relative humidity (*P. dilatatus* at 70% RH) was there a significant difference between 13.5 and 18.5 °C (Fig. 3). In contrast for all four species/temperature combinations mortality was significantly higher at 70% RH than at 90% RH, again suggesting a more consistently higher response to a 20% decrease in relative humidity than for responses to a 5 °C increase in temperature.

Discussion

In this paper we use a reductionist, experimental approach to evaluate different responses to components of climate change. Specifically we address the question: will responses to climate change by soil animals indirectly help to mitigate the direct effects of increased soil temperatures increasing microbial activity and hence microbially mediated CO_2 emissions? These direct responses of micro-organisms to a change in global temperatures could potentially cause a positive feedback between increased

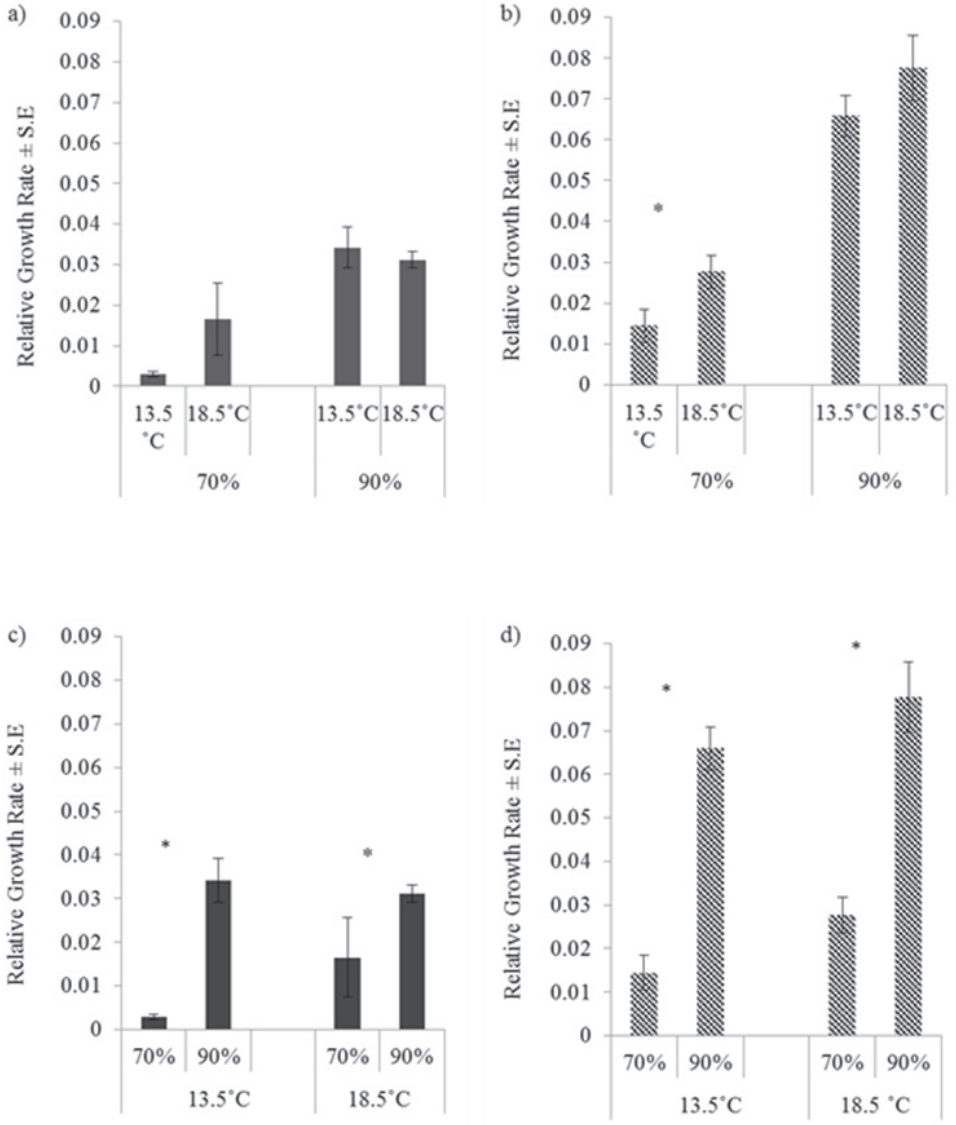

Figure 2. Responses of relative growth rates to temperature and relative humidity. Responses to differences in temperature by a) *Oniscus asellus*, ($F_{1, 36}$ = 0.905, P = 0.348) and. b) by *Porcellio dilatatus*, ($F_{1, 36}$ = 5.112, P = 0.030); to differences in relative humidity of c) *Oniscus asellus*, ($F_{1, 36}$ = 17.125, P < 0.001) and d) *Porcellio dilatatus*, ($F_{1, 36}$ = 84.326, P < 0.001). Asterisks denote differences signficance at P < 0.05.

atmospheric CO_2 concentrations, increased greenhouse effects, and increases in soil temperature and microbial metabolism (Reichstein et al. 2002).

While predictions of above ground temperature and rainfall patterns are reaching a sophisticated level of both spatial and temporal resolution (IPCC 2013), predicting how these changes will affect the micro-climate of the soil/litter interface inhabited by

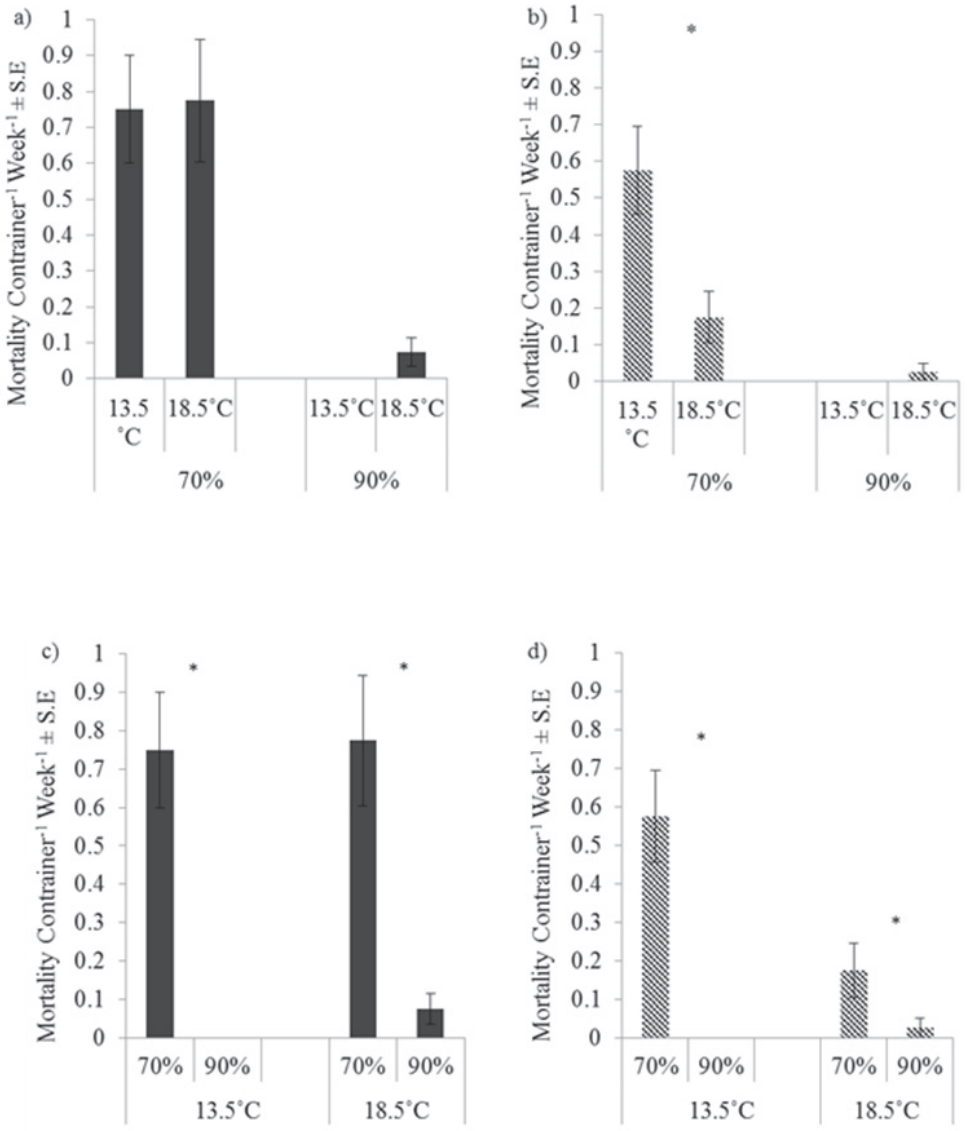

Figure 3. Response of mortality to temperature and relative humidity. Responses to temperature by a) *Oniscus asellus*, (U = 3097.0, P = 0.640. and b) by *Porcellio dilatatus*, (U = 2254.5, P = 0.016) and to relative humidity by c) *Oniscus asellus* (U = 1851.5, P < 0.001) and d) by *Porcellio dilatatus* (U = 2277.5 P < 0.001). Asterisks denote differences signficance at P < 0.05.

many soil animals, is less precise due to the buffering effects of the cooling capacity of the soil and evaporation of water from within it. In order to balance obtaining significant signal to noise ratios while retaining a realistic approach to predicted changes, we have used the top end of the range of predicted increases in air temperature in the 21st century for high emissions scenarios (5–6 °C) (Bale et al. 2002) and, in the absence of

reliable predictions of future changes in relative humidity at the soil surface, reductions in relative humidity of 20–25% based upon observed intra-annual ranges in relative humidity in the litter layer of a fixed dune grassland (Hassall 1976). A 50% reduction of summer rainfall is predicted for south east England by 2090. The period between future summer events is also predicted to double producing longer droughts (Murphy et al. 2009). Moss (2007) found that reducing simulated rainfall in experimental mesocosms by 50% reduced soil moisture by 20%.

Isopods are used as model arthropod macro-decomposers because their physiological, behavioural and ecological responses to different microclimates are well known (Sutton 1980) and they are prominent components of the macro-decomposer fauna in many ecosystems (Davis and Sutton 1977). However there is a wide range of adaptation to the terrestrial environment between different families of the sub-order Oniscidae (Edney 1965, Warburg 1968), members of the family Oniscidea notably having much less well developed respiratory surfaces than the pleopodal lungs characteristic of members of the family Porcellionidae.

The experimental animals responded to both an increase in temperature from 14 to 20 °C and a reduction of 25% in relative humidity by increasing the degree of aggregation, as found over other temperature and relative humidity ranges (Allee 1926, Hassall et al. 2010). These components of the functional responses of isopods to predicted changes in temperature and rainfall will thus act together to reduce isopod activity, which will therefore reduce the extent to which these soil animals are likely to act as "Prince Charming" in Lavelle and Spain's (2001) "Sleeping Beauty" analogy of how soil animals stimulate micro-organisms by transporting propagules to new substrates. Aggregative responses of isopods to predicted trends in climate change are therefore likely to partially mitigate the positive feedback cycle of increased soil temperatures and CO_2 emissions.

Both growth and mortality rates responded consistently more to a 20% decrease in relative humidy than to a 5 °C increase in temperature. In three of the four species × humidity treatments growth rates did increase over this range of temperature, as would be expected from previous studies of isopod growth rates (Grundy and Sutton 1989) but only one of the increases was significant. In contrast for all four species × temperature comparisons a decrease of 20% in relative humidity resulted in significant decreases in relative growth rates. The same pattern was apparent for mortality rates as only one out of four changes in mortality rates in response to an increase of 5 °C was significant which was an anomalous decrease in mortality at the higher temperature. In contrast mortality responses to decreases of 20% in relative humidity were consistently significant in all four temperature × species combinations, higher mortality occurring at the lower relative humidity as found in other studies summarised by Sutton (1980). As fecundity is a function of size in terrestrial isopods (Sunderland et al. 1976) the net effect of a combination of responses for growth and mortality on the overall numerical response by isopods to climate change is therefore likely to be negative and so likely to reinforce the aggregation functional responses in reducing stimulation of soil micro-organisms. Indirect effects of climate change, via effects on stimulation of soil micro-organisms by these soil animals altering their regulation of microbial activity, would

therefore reinforce the mitigation of increased microbial metabolism and hence CO_2 emissions from soils likely to result from predicted future increases in temperature.

In conclusion the answer to the question of whether the net functional and numerical responses of these model arthropod macro-decomposers will have a negative effect on the potential positive feedback between increased soil temperatures, increased soil CO_2 emissions increasing greenhouse warming and hence further soil warming, will vary with regional differences in future rainfall patterns. In regions predicted to experience significant decreases in levels or periodicity of rainfall, the functional role of the animals in stimulating the micro-organisms is likely to be reduced. This can be predicted to partly mitigate against the potential positive feedback that could lead to increased CO_2 emissions from soils.

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Personality affects defensive behaviour of *Porcellio scaber* (Isopoda, Oniscidea)

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Abstract

We evaluated individual behavioural patterns of isopods expressed as tonic immobility following some intrusive treatments. Common rough woodlice, *Porcellio scaber*, were kept individually in plastic boxes and tested for tonic immobility repeatedly. Reactivity, sensitivity (number of stimuli needed to respond), and endurance of tonic immobility (TI) according three types of treatments (touch, squeeze, drop) were evaluated. Touch was the weakest treatment and it was necessary to repeat it a number of times to obtain a response; while squeeze and drop induced TI more frequently. Nevertheless, duration of the response persisted for a longer time with the touch treatment. Within each set of the three treatment, the strongest response was the third one, regardless of treatment type. Duration of reaction was affected by the size of the woodlouse, the smallest individuals feigning death for the shortest time. Despite body size, we found a significant individual pattern of endurance of TI among tested woodlice, which was stable across treatments as well as across time (5 repetitions during a 3 week period). *Porcellio scaber* is one of the first species of terrestrial isopods with documented personality traits.

Keywords

Anti-predatory behaviour, death feigning, thanatosis, predation, behavioural trait

Introduction

Generally, when animals encounter their predator they (1) run away, (2) attack it or (3) stay invisible and/or look unpalatable.

Anti-predatory behaviour including boldness can be a part of animal personality. Personality of animals has been routinely studied during the last twenty years, although the study of personality in vertebrates prevails. Behavioural traits, which are consistent over time in individuals and as a response to different situations, have been described as a personality (Reale et al. 2007). The concept of personality has been used for a relatively broad spectrum of invertebrates including crustaceans (e.g. Briffa 2013, Biro et al. 2014, Brodin and Drotz 2014), but not explicitly studied in terrestrial isopods previously. The main behavioural traits found in Crustacea are boldness (Brifa et al. 2008; Hazlett and Bach 2010; Brifa and Twyman 2011; Brifa 2013), voraciousness (Biro et al. 2014) and activity (Yli-Renko et al. 2014).

Change in anti-predatory behaviour during growth and development of animal can challenge stability over time of the behavioural traits mentioned above. Examination of animal personality traits must consider consistency over two different time intervals: short intervals to determine whether behaviour is sufficiently consistent to be included in a study of personality, and longer intervals to determine how behaviour changes over the course of a lifetime (Stamps and Groothuis 2010).

During their evolution, terrestrial isopods colonised land and they were faced with new types of stresses, including new types of predators (Broly et al. 2013). The anti-predator mechanisms used by woodlice include escape, armour, cryptic colouration, chemical protection, acoustic warning, feigning death and/or specific posture (Witz 1990). Some of these strategies are not direct adaptations against predators, but evolved as parts of their terrestrial life-style. For example, escape is simply an extension of the ability to move as necessary to find food and mates, while armour is usually found in isopods living in (semi) dry conditions, which need to minimize water loss using thick cuticle (e.g. Smigel and Gibbs 2008; Csonka et al. 2013) and evolved as a defensive reaction. Chemical defensive secretions are a direct adaptation against predators being at least spider- (Gorvett 1956) and ant-repulsive (Deslippe et al. 1996; Yamaguchi and Hasegawa 1996).

Terrestrial isopods developed behavioural protection known as tonic immobility or death feigning, which is related also to behaviour known as "taking specific posture". In general, the main difference between these categories is that "taking posture" is aimed for protection against being swallowed by a predator (e.g. Honma et al. 2006) and "feigning death" increases the probability to be ignored by predators with sight as the prevailing sense. This behaviour includes the so-called conglobation or volvation, behaviour typical for members of some isopod families such as Armadillidae, Armadillidiidae, or Cylisticidae, as well as for pill millipedes (Glomerida) and giant pill millipedes (Sphaerotheriida), some soil mites (Oribatida), and cuckoo wasps (Chrysidoidea). Conglobation involves the body being rolled into non-perfect or perfect ball with legs,

antennae and ventral body surface more or less hidden. Non-perfect conglobation (e.g. typical for the genus *Cylisticus*) is less effective as uropods and antennae are not well protected. Nevertheless, tonic immobility is a much more general behaviour than conglobation and it is used by isopods (Quadros et al. 2012). Tonic immobility in non-conglobating forms of isopods is characterised by the contraction of the body and the contraction and folding of the legs towards the ventral side while holding the antennae folded or extended backwards and pressed against the dorsal part of the first pereonites (see fig. 1 in Quadros et al. 2012). During this posture the organism lacks motional responsiveness to external stimulation. Differences between death feigning and conglobation (called also shrinking in Anura) were discussed in the case of amphibians, but for isopods these differences are of marginal importance (Toledo et al. 2010).

The usefulness of feigning death as an anti-predatory behavioural strategy can theoretically be dependent on body size of an animal. If smaller animals can be easily overlooked by predator, the frequency of using this strategy by small animals can be higher than by bigger animals. This pattern was confirmed in some studies (Hals and Beal 1982; Quadros et al. 2012) but not found in other ones or in other species (Hazlett and Bach 2010; Quadros et al. 2012) for several crustaceans including terrestrial isopods.

We studied anti-predatory behaviour of the Common rough woodlouse *Porcellio scaber* Latreille, 1804, and we added a new parameter to standard experimental design – repetitions at the individual level. With this modification we were able to study the stability of behavioural traits, i.e. animal personality. The main aims of this research were: Are there any patterns in death feigning (tonic immobility, TI) behaviour? Is TI affected by type of treatment or its order? Is there a body-size pattern of behaviour among woodlice suggesting any developmental changes of its behaviour? Despite size of body, is there an individual specific pattern of behaviour among woodlice, i.e. are we able to evaluate their boldness on personal level?

Methods

Subjects and housing conditions

Several hundreds of Common rough woodlice, *Porcellio scaber*, were collected in the environment of Kutna Hora, Czech Republic (urban green areas and gardens) during June 2013. Following transport to laboratory, they were not sexed, but sorted in three size categories by length (small < 7 mm, medium 7–12 mm, and large > 12 mm). Size of woodlouse is related to its age (Zimmer 2002). Fifty individuals of each size category were inserted into small non-transparent plastic boxes (area 33 cm^2) each with a thin plaster of Paris layer on the bottom. Each isopod individual had its own identifying code (ID) marked on its box. These codes enabled analyses of the stability of its behaviour (personality). Isopods were fed on potatoes and plaster was kept moist; natural (room) temperature regime was maintained at 21–26 °C.

Procedure

Behavioural experiments followed the design used by Quadros et al. (2012); each isopod was exposed to several treatments. One experimental set contained three types of treatments to induce tonic immobility (TI): touch, squeeze, and drop. The touch stimulus was applied as gentle nudge to the isopod with forceps. The squeeze stimulus was applied as a firm grab to the isopod body by entomological soft-metal forceps, when one prong was undercutting the ventral part of the body and the other part was applied on the dorsal part. The drop stimulus was similar to squeeze one, though followed by lifting to *ca* 10 cm and then letting it drop back in the box.

The first treatment was applied and if TI was induced, its duration was measured. If necessary, the stimulus was repeated up to 5 times in order to induce TI. If TI was not induced, lack of reaction was recorded. We let individual woodlouse rest for approximately 30 minutes and applied the second treatment in the same way and the third treatment after a further half hour, respectively (Fig. 1). ID of woodlouse, order of types of treatments, sensitivity or promptness of TI induction (i.e. number of stimuli needed) or non-reactivity; and endurance of TI (i.e. time from start of TI to the first movement of antenna or leg) was measured in each experimental set. Each individual was involved in five experimental sets with 4 day intervals between experimental sets. The order of stimuli was changed systematically to distinguish the effect of type of stimulus from an effect of order of stimuli.

Data analysis

We tested the effects of the three types of treatment (touch, squeeze and drop) on reactivity (presence/absence of reaction to stimulus, i.e. probability of inducing TI), sensitivity

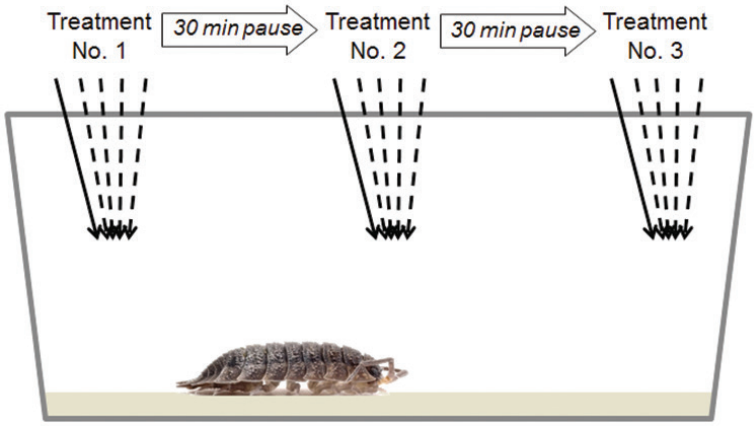

Figure 1. Design of one experimental set. Dashed arrows symbolise repeated stimuli applied if previous stimulus did not evoke tonic immobility. Experimental sets were applied repeatedly over a three week period; each individual was exposed to five experimental sets with 4 days intervals between.

(number of stimuli needed to induce TI) and endurance of TI. Experimental sets which failed to induce TI were excluded from next data analyses. To determine the effect of different types of treatments we conducted repeated measures ANOVA. The error term of ANOVA reflects that we had the type of treatment nested within individuals of woodlice (ID). Data were not normally distributed therefore we transformed data by decimal logarithm. For multiple comparisons we used a pairwise t-test with adjusted p-values by the Holm correction. We used the F test to check the significance of the explanatory variables. Kendall's coefficient of concordance was computed in order to determine the consistency of between-individual differences in the three types of treatment. We also used the correlation of TI endurance among different type of treatment. Significance of correlations was tested by using Kendall method with Bonferroni correction.

Results

Three isopods died after the first experimental set, but data are available to evaluate from 738 experimental sets; TI as a reaction to at least one treatment was recorded in 334 sets (45% of sets) in 35 woodlice (23% of individuals). TI was induced by all treatments during the same experimental set in 41 experiments (6%) in 25 woodlice, with only one individual showing TI at each of the 15 treatments (i.e. through all five experimental sets).

If a woodlouse reacted to a treatment in an experimental set, the probability of reaction was influenced by type of treatment ($F_{2,298}$ = 1165.00, $p < 0.001$, Fig. 2b); in those experimental sets isopods reacted to drop and squeeze in all cases, but to touch in *ca* 20% only. If isopods reacted to treatment by TI, duration of TI significantly depended on the type of treatment ($F_{2,298}$ = 2.97, $p = 0.052$, Fig. 2d), too: with touch followed by the longest TI. Also reactivity, i.e. number of stimuli needed to induce TI, was significantly dependent upon the type of treatment ($F_{2,298}$ = 517.00, $p < 0.001$, Fig. 2f); if the global probability to react to touch is the lowest, more stimuli of touch were necessary to induce TI.

To avoid misunderstandings relating to the effect of treatment type and its order in the experimental set, the order of the applied treatments was changed. Without respect to type of treatment, the third treatment was the most probable to be followed by TI ($F_{2,298}$ = 81.00, $p < 0.001$, Fig. 2a). Nevertheless the endurance of TI shortened significantly during experimental sets ($F_{2,298}$ = 9.63, $p < 0.001$, Fig. 2c). On the other hand, number of stimuli needed to induce TI was significantly related to the order of the treatment ($F_{2,298}$ = 16.55, $p < 0.001$, Fig. 2e).

Although there were no significant differences among body-size categories of *P. scaber* in the probability of inducing TI ($F_{1,148}$ = 0.73, $p = 0.395$), the longest TI duration was performed by medium body sized woodlice ($F_{1,148}$ = 6.75, $p < 0.05$, Fig. 3).

Personality, i.e. individual stability of duration of TI was confirmed by Kendall's concordance analysis for the whole reactive group of isopods ($W = 0.73$, $p < 0.001$); there were individual patterns of endurance of TI irrespective of type of treatment or

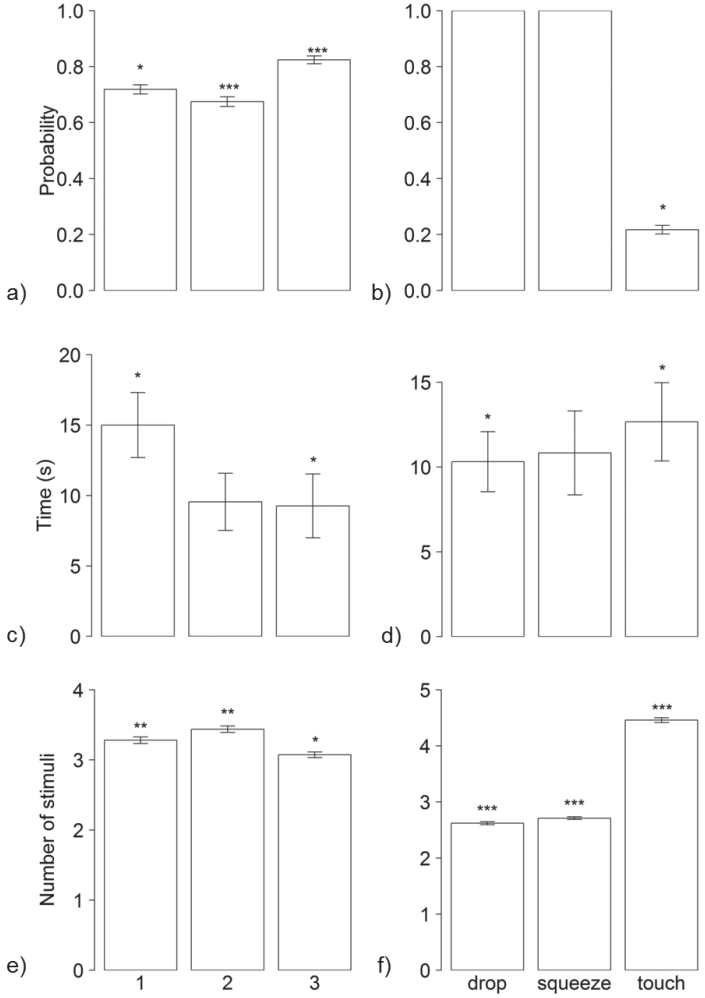

Figure 2. Tonic immobility of *Porcellio scaber* induced by different treatments: **a** probability of inducing TI by the first, the second and the third treatment **b** probability of inducing TI by different treatments **c** endurance of TI following the first, the second and the third treatment **d** endurance of TI following different treatments **e** sensitivity, i.e. promptness of inducing TI by the first, the second and the third treatment **f** sensitivity, i.e. promptness of inducing TI by different treatments. (*** $p < 0.001$; ** $p < 0.01$; * $p \leq 0.05$)

Table 1. Correlations between durations of TI of *Porcellio scaber* induced by different treatments: D – drop, S – squeeze, T – touch. (*** $p < 0.001$; ** $p < 0.01$; * $p \leq 0.05$)

	all animals			**large-size animals**			**medium-size animals**			**small-size animals**		
	D	**S**	**T**	**D**	**S**	**T**	**D**	**S**	**T**	**D**	**S**	**T**
D	-	0.55***	0.45***	-	0.40*	0.32*	-	0.71**	0.56**	-	0.44*	0.44*
S		-	0.49***		-	0.61*		-	0.52**		-	0.32**
T			-			-			-			-

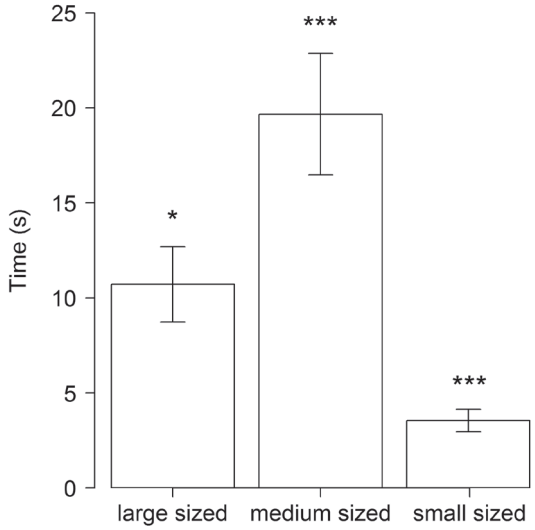

Figure 3. Endurance of tonic immobility of *Porcellio scaber* of different body sizes induced by treatments. (*** $p < 0.001$; ** $p < 0.01$; * $p \leq 0.05$)

its order. To avoid obfuscation of personality and size-dependent differences in behaviour, concordance analyses for individual size categories were calculated and revealed significant stability of endurance of TI inside all body-size categories (large size: $W = 0.68$, $p < 0.001$; medium size: $W = 0.82$, $p < 0.001$; small size: $W = 0.65$, $p < 0.001$). Stability of patterns of durations of TI can be visualised by correlations between endurances of different TI values (Fig. 4). Correlations between duration of TI were significant for *P. scaber* analysed as a whole group as well as between different body-size groups (Table 1).

Discussion

We evaluated reactivity, sensitivity, and duration of tonic immobility of *Porcellio scaber*. It is difficult to evaluate the functional significance of anti-predatory behaviour, as there are several interfering behaviours which affect probability of an animal being recognized, captured and consumed by predators (Lind and Cresswell 2005). These behaviours can have evolved independently; nevertheless it is impossible to measure the independent effect of one of those behaviours on the fitness of target animals. For this reason we cannot say that tonic immobility increases the fitness of *P. scaber*.

Generally, the reactivity was relatively low (23% of isopods). It is known that tonic immobility is not the main anti-predatory strategy for *P. scaber*, as a clinger ecomophological type (Schmalfuss 1984). They use sticking against a surface, or run away more frequently to escape predators (77% of woodlouse in our study tried to run away exclusively), or uses chemical protection (Gorvett 1956; Deslippe et al. 1996).

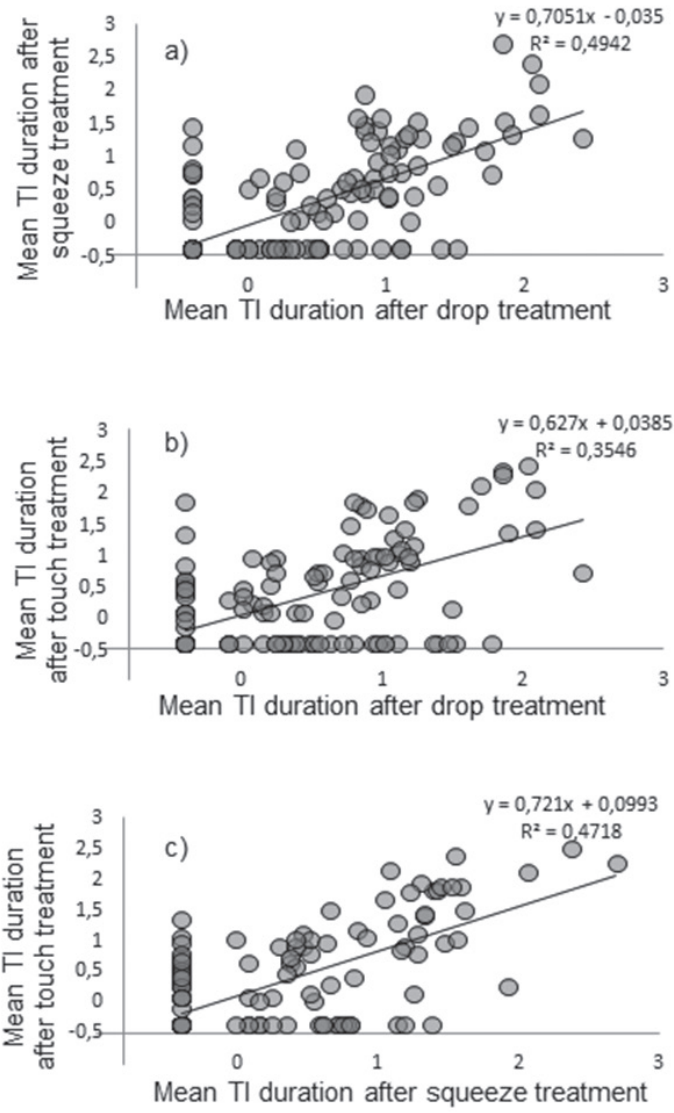

Figure 4. Correlations between duration (in seconds) of TI of *Porcellio scaber* induced by the different treatments: **a** correlation between duration of TI induced by squeeze and drop **b** correlation between duration of TI induced by touch and drop **c** correlation between duration of TI induced by touch and squeeze. Data were transformed by decimal logarithm.

Nevertheless, Quadros et al. (2012) found *Porcellio dilatatus* to be a highly responsive species (89% specimens used TI).

Reactivity of isopods was affected by the type of treatment. Whereas drop and squeeze were followed by TI regularly, touch was not an effective treatment for TI in some specimens. The explanation can be found in the manipulation of isopods by dif-

ferent kinds of predators (e.g. Sunderland and Sutton 1980, Dejean 1997, Řezáč and Pekár 2007, Quadros et al. 2012). Despite the lack of experimental verification of tonic immobility as defence behaviour against predators and regarding the size of *P. scaber*, we can hypothesise that drop treatment is probably more similar to manipulation by some vertebrate visual predator (birds, amphibians, or lizards). Squeeze can be similar to manipulation by some small vertebrate or large invertebrate predators (e.g. small rodents or shrews, ground beetles of the genus *Carabus*), whereas touch resembles the manipulation of small invertebrate predators (spiders, centipedes, ants, etc.). According to these categories of predators, TI following drop can be very useful, if the isopod is lost by predator in leaf litter. Big predators do not loose time for looking for one small prey item. They probably continue walking and searching for another prey. Similarly, TI as response to squeeze can also help the attacked isopod to survive, if the predator is not able to manipulate the immobile prey very well. By contrast, the most effective defensive strategy against small invertebrate predators is the secretion of chemicals (Deslippe et al. 1996), which may not be so effective against larger predators.

It is necessary not to forget that *P. scaber* is strongly thigmotactic (e.g. Friedlander 1964) and lives in large aggregations (Broly et al. 2012): this is important for two aspects concerning its TI reaction. First, touch is a common stimulus in the way of life of woodlice. In aggregates, there are many conspecific individuals around; reacting by TI to each touch becomes meaningless. For this reason, low reactivity and low sensitivity to touch is understandable. But if touch is repeated several times (it was necessary to repeat it more times than drop or squeeze), endurance of TI is longer than TI following drop or squeeze. This is probably because of the foraging mode of the predator: small invertebrate predators such as spiders or ants can manipulate small isopods for a longer time and can wait for the first movement (providing time to attack the un-armoured ventral side). Larger predators do not waste time by waiting; they swallow prey immediately if they notice and catch it.

Another advantage of aggregates is the higher probability of being passed over by a predator among running conspecifics (Miyatake et al. 2009). If larger predator turns over the shelter of a group of isopods (e.g. stone or bark on dead stump), it can be useful to stay in TI and wait until the predator is lured away by other, running members of the aggregation. It can be gainer strategy even if the woodlouse is lost by the predator (drop or squeeze). In addition, the shorter duration of TI can be more useful if the lured-away predator is coming back to search for the last prey items. It can be an explanation for higher reactivity and shorter endurance for TI following drop and squeeze stimuli.

Studies have shown changes in behaviour according to the type of disturbing treatment. Carbines et al. (1992) studied the character of escape mechanisms of isopods from predators. They measured turn alteration in a simple labyrinth and related it to the probability of survival (as direction of run). If the treatment was harmless cotton-wool fluff, the probability of survival was much lower than if *Dysdera* spider predators were the agent of disturbance. It means an authenticity of stimulus affected its defensive behaviour; perhaps over time in our prolonged experiment the authenticity

of disturbance was decreasing. During one experimental set in our study, the reactivity increased in the third treatment while in the third treatment duration of TI was reduced. This resembles a situation when the isopod is (hypothetically) able to evaluate the meaningless stimulation of the experimenter and learn "to escape" from this situation by a more prompt TI response for a shorter time. As this "explanation" is rather implausible, shorter duration of TI in the last stimulus can be explained also by quick habituation of *P. scaber* to stable environmental cues, as was described by Anselme (2013). Habituation, i.e. changes of response to repeated stimulus was reported also for the aquatic crab *Chasmagnathus granulatus* (Tomsic et al. 2009).

Although our research is not the first to look into TI in terrestrial isopods, the results presented here enable to test repeatability of responses of individual isopods, i.e. its personality. The concept of personality was used for behavioural studies of some Crustacean species, mainly Decapoda, i.e. in crabs, hermit crabs, crayfishes (e.g. Briffa 2013, Biro et al. 2014, Brodin and Drotz 2014), as well as Isopoda (Yli-Renko et al. 2014). Among terrestrial isopods, the only study dealing with personality known to the authors was done by Matsuno and Moriyama (2012). They found a correlation between the walking speed and endurance of conglobation in some specimens of the Common pill bug *Armadillidium vulgare*. Nevertheless, this "stable internal factor" was found only in specimens showing a stable-style end of conglobation: specimens that finished conglobation in two trials by leg movement or antenna movement consistently, were more "brave" (shorter duration of tonic immobility) and ran faster compared to leg-antenna "alternators" (i.e. specimens ending conglobation by antenna movement and leg movement in two trials). We also found correlations in individual specimens for duration of TI across different types of treatment and these correlations were found over three weeks (five experimental sets with 4 day breaks), meaning that there were some consistently more "bold" woodlice (short TI) and some more "shy" woodlice (long TI).

Correlations between length if TI, even if there is a decrease of endurance of TI during one experimental set, can be caused by habituation of isopods to repeated treatment as well as their sensitivity to new type of treatment. Anselme (2013) found that *P. scaber* individuals are able to habituate to an environment in around 10 minutes. Over this time, they become less interested in stable stimulus and their activity decreased. In the same study woodlice preferred new stimuli (such as a new texture of substrate) if it was provided (Anselme 2013), or a random pattern of known stimuli (Anselme 2015), so there is some evidence of "curiosity" in *P. scaber* (although not studied at individual level).

Documented "boldness", as a parameter of personality of *P. scaber*, is independent of size (age) of specimen. Hals and Beal (1982) reported that the largest specimens (> 1 cm of length) of *P. scaber* reacted by TI at less intensity compared to smaller specimens (< 1 cm). Similarly Quadros et al. (2012) found the same pattern in reactivity for *Balloniscus sellowii*. However we did not find significant differences in reactivity of woodlice in our three body-size groups, although there are differences in endurance of TI among groups. The longest reaction time was measured in medium-sized woodlice

(7–12 mm) and shortest in small-sized woodlice (< 7 mm). One explanation could be sought in terms of changes of the effectiveness of TI as a defence mechanism against predators. TI is not necessary for large woodlice against medium-sized and smaller predators, because large woodlice are less catchable and can use chemical defence: their glands are well developed and able to produce sufficient amount of secretions (Gorvett 1956, Sutton 1970) in comparison to the less developed glands in smaller stages of *P. scaber*. As well TI would not be a successful protection for the smallest woodlice against predators such as *Carabus* or centipedes, as they are easy to manipulate. Indeed, the mortality of juvenile stages of isopods is estimated to reach 80% (Sutton 1970) and 11–51% decrease in populations is caused by predation upon juveniles by invertebrates (Sunderland and Sutton 1980). This indicates TI can be a useful strategy mainly for medium-sized *P. scaber* specimens.

Besides finding differences in endurance of TI between body size groups, we also identified personal behavioural patterns in all tested individuals, as well as variation within these body-size groups. These findings are not able to resolve if personality is changing during individual development or not. Although behavioural traits can be stable across short time intervals, changes to personality due to development can cause inconsistency in responses to stimuli over longer time intervals (Stamp and Groothuis 2010). We did not evaluate if traits remained the same over long time intervals, but this type of stability was not proved for marine isopod *Idothea baltica* recently (Yli-Renko et al. 2015). Investigation of long-time stability of behavioural traits in terrestrial isopods should be a possible goal of future studies.

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NEIGHBOUR-IN: Image processing software for spatial analysis of animal grouping

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Abstract

Animal grouping is a very complex process that occurs in many species, involving many individuals under the influence of different mechanisms. To investigate this process, we have created an image processing software, called NEIGHBOUR-IN, designed to analyse individuals' coordinates belonging to up to three different groups. The software also includes statistical analysis and indexes to discriminate aggregates based on spatial localisation of individuals and their neighbours. After the description of the software, the indexes computed by the software are illustrated using both artificial patterns and case studies using the spatial distribution of woodlice. The added strengths of this software and methods are also discussed.

Keywords

Inter-individual distances, aggregation, individual attraction, spatial distribution

Introduction

Group formation or crowd formation behaviour occurs in many taxa, from very simple organisms (bacteria) to highly complicated organisms (e.g. mammals), both wild and domestic (Krause and Ruxton 2002; Parrish and Edelstein-Keshet 1999; Parrish and Hamner 1997). Depending on the species, crowds of individuals are referred to as a

herd in mammals, flocks in birds, schools in fish, swarms in insects and many other terms that indicate aggregation. Species can be range to simply aggregated species with temporal fluid group composition to very complex and relatively stable composition with non-random structures (Kutsukake 2009).

Group living confers several advantage compare to solitary lifestyle and many animals live in groups for part or all of their lives. Aggregate can be the results of abiotic factors and environmental heterogeneity (non-social aggregates) or relied on mutual attraction (interattraction) resulting in different formation process (Broly et al. 2013). In most of the cases, the ecological and social factors that explain group life are unknown. Ecological factors favouring group life are numerous and usually addressed the benefits from individuals association or differences in fitness related with spatial position of individuals in groups (Krause and Ruxton 2002). The main two factors are the availability of food and the presence of predators and the optimum group size change according to the species and environmental pressure variations. For example, group's dimension and geographical repartition change according of both predators and food availability in starlings (Zoratto et al. 2009). Group size reduces the risk of predation (Brown and Brown 1987) and offers a better foraging efficiency (Stacey 1986) and large groups present feeding advantage compared to small groups (Miller and Dietz 2006). Little is known in general about how group size affects individual welfare (Ohl and Putman 2014). Costs are mainly the result of competition for limited resources (food, mate, habitat...) and increase in group size favours disease transmission, probability of infection and ectoparasitism that affect survival (Brown and Brown 2004; Krause and Ruxton 2002).

Animal group formation is a complex dynamic system made up of potentially thousands of individuals. The group formation is the result of each individual's behaviour under the influence of many (and non-exclusive) parameters, such as heterogeneities of environment, inter-individual interactions, and temporal changes (ie, season, reproduction, and feeding) (Camazine et al. 2003; Sumpter and Pratt 2009). In cockroaches, the aggregation relies on mechanisms of amplification depending on the interactions with other individuals (Jeanson et al. 2005). Group dynamic is also the result of the heterogeneous social relationships and conflict management for maintaining group living in mammals (Kutsukake 2009). Moreover, animal groups exhibit different patterns according to the species (Parrish and Edelstein-Keshet 2000; Parrish and Edelstein-Keshet 1999) which necessitate incorporating species characteristics for conceptual questions and modelling.

To better understand group dynamic complexity, the identification of factor influencing group composition and how the dynamic change, it's necessary to provide specific tools.

Many analytic models and simulation of aggregation (mathematical and computer-based) offer interesting tools to investigate aggregation phenomena in various species (Schellinck and White 2011), including scale of different aggregate-level behaviour (individuals, castes, groups, or species).

Description of animal movements in their environment is necessary to understand species, dispersion strategy as results of individual intrinsic factors, collective responses and social relationship (including change of individual composition moving in or out the group) and also to predict their geographical needs and spatial distribution, providing consistent data for models and simulation accuracy (Schellinck and White 2011).

In order to study aggregation patterns, researchers can use various methods for data collection that could be divided into three mains categories (1) manual : observation and capture (Krause et al. 2000; Spieler 2003), photography and film (Aschwanden et al. 2008; Ballerini et al. 2008; Boulay et al. 2013; Eklund and Jensen 2011; Le Goff et al. 2009; Yoshida et al. 2010) (2) semi-manual : sonar and echo sound (Axelsen et al. 2001; Gerlotto et al. 2006; Gerlotto and Paramo 2003; Handegard 2007; Soria et al. 2003), (3) automatic : microtransponders (Jeanson 2012), RFID tagging (Planas-Sitja et al. 2015).

In the current study we propose a data processing software called NEIGHBOUR-IN which allows spatial coordinates to be attributed to individual (object) of up to three different categories (groups), such as species, sex, age, size, moult stage, etc. We illustrate the software applications with artificial patterns and experimental study of aggregation in terrestrial isopods (Crustacea, Oniscidea). The software then calculates indexes qualifying spatial distribution, composition of groups and many other parameters. All data outputs can be used for further analysis and quantification of spatial variations.

Methods

NEIGHBOUR-IN is a software designed to analyse individuals' coordinates belonging to up to three different groups. The software can be used only for picture analysis and not for film analysis. However film screenshots in the appropriate format could be made to follow the dynamics of the studied processes. Once the image of the objects (i.e. individuals) is loaded, the user identifies each individual by clicking on anterior and posterior extremities. Each object belongs to up to three different groups. The software includes statistical analysis and indexes to discriminate aggregates based on spatial location of individuals and their neighbours. At the end of the treatment the table of coordinates can be exported for further spatial analysis. Different displays are available in order to show the aggregates or the influence distance of each object (for example the area where the antennas can touch and interact with another individual in the case of insects).

Software installation and procedures (Fig. 1) are detailed in a separate additional document (NEIGHBOUR-IN Reference Manual). In the current article we will define and illustrate the different indexes of inter-individual distances and spatial distribution provided by the software and how such indexes can be helpful in the analysis of spatial distribution of objects in general and animal aggregation in particular.

Three categories of output characterising the group structure and for statistics analysis are prepared: i) inter-individual distances; ii) aggregation profile; and iii) spatial distribution.

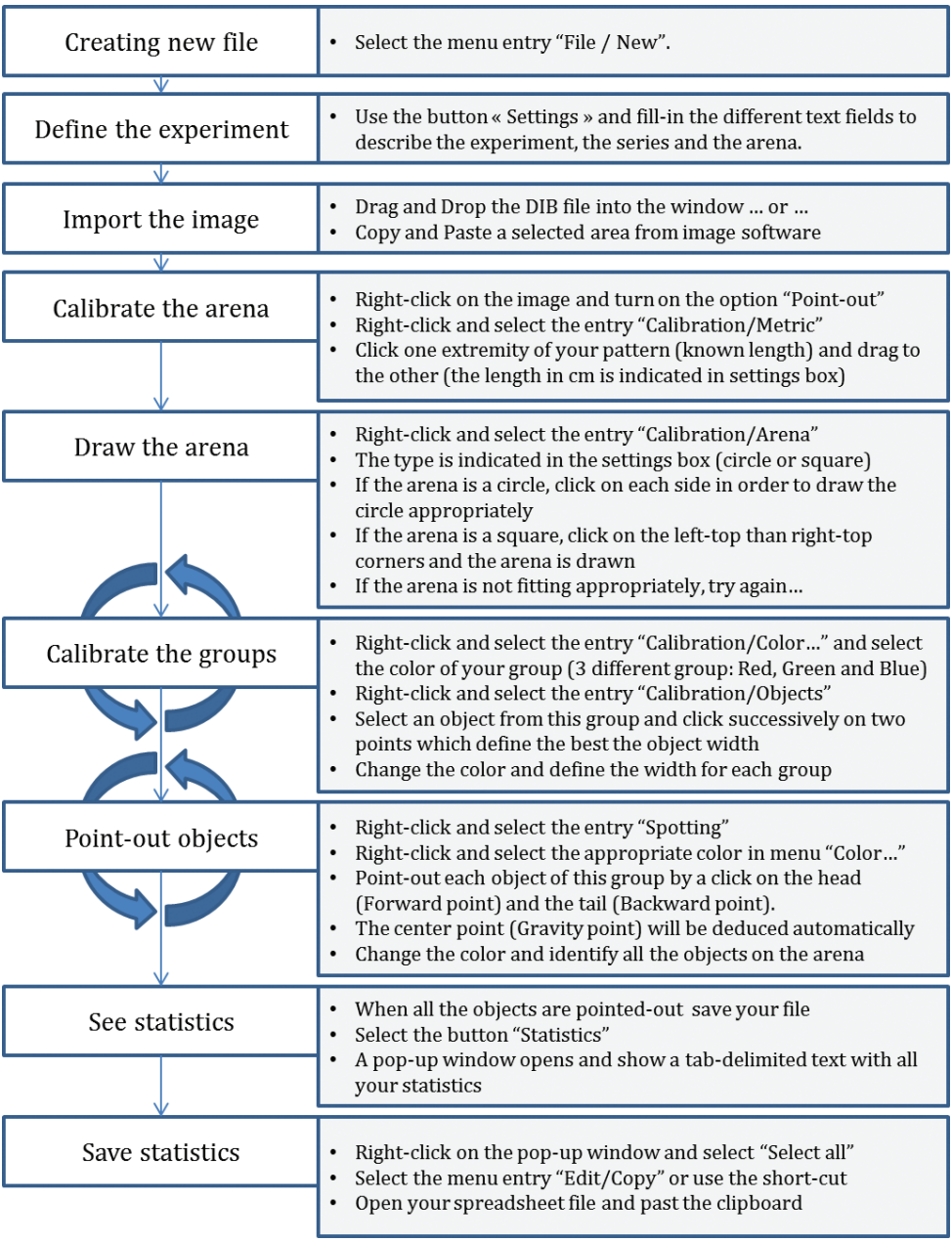

Figure 1. Flow chart of the creation of a new NEIGHBOUR-IN file. This figure presents the different steps in the creation of a new file, from the importation of the snapshot to the calculation of the statistics of dispersion.

The indexes and data included in the statistics report are:

Header: all the parameters of the experiment (file name; image size in pixels; experiment name; total number of objects identified; calibration information with the ratio "pixel / centimetre", width or radius of the box and number of cells; and for each group: name, number of objects, mean body width).

Inter-individual distance: the mean inter-individual distances between the centre-point (G point) of all objects and both for each inter-group and intra-group combination are provided. In addition to the mean value, descriptive statistics are available in the output (sample size, standard error, minimum and maximum).

Distance to nearest neighbours: these descriptive statistics are identical to the ones described in the previous section (same inter-individual distances) but only between nearest neighbours (belonging to the same or different groups). The number of neighbours is defined by the user in the parameters dialog box.

Statistics on aggregates: When at least one of the three points of an object is included within the perception field of another object, both are considered aggregated. The perception field is calculated using the mean body width of the group multiplied by the perception ratio determined by the user for the group. The statistics include the number of aggregates automatically identified by the software; number and composition (type of group) of isolated objects; composition of each aggregate; percentage of aggregation in general and for each group (*% Aggr.*); and in the case of heterogeneous populations, Aggregation Heterogeneity Index (*AHI*). The *AHI* provides an estimate of the homogeneity of subpopulation distributions. It will be maximum (1) when the aggregates are "pure" (i.e. each one is composed by objects which belong to the same group) and minimum (0) when all individuals in the aggregates are equally mixed whatever their features. The index is calculated using the following formula:

$$AHI = \frac{\sum_i^N m_i}{M/\ 2}$$

where N is the number of aggregates; mi is the minimum number of objects belonging to the same group for the aggregate i; M is the total number of objects aggregated.

Statistics on distribution: The area is divided in different cells by the software, defining a grid. The distribution of the objects on the area is described by two indexes: the Spatial Distribution Index (*SDI*) for each group, and the Spatial Mixed Index (*SMI*) for groups' pair comparison.

Spatial Distribution Index (*SDI*) is given for each group and is calculated by dividing the number of cells/sectors where at least one object of the group is present by the total number of objects in the group. *SDI* will be maximum (1) when each object is in a different cell and minimum when the objects are aggregated in few cells (0.125 for eight objects in the same cell). Therefore the *SDI* index is sensitive to

the number of cells (user defined) and the size of the objects (i.e. how many objects can contain a single cell).

Spatial Mixed Index (*SMI*) is calculated for two groups by dividing the number of cells in which at least one object of both groups is present by the total number of cells occupied by the two groups. *SMI* will be maximum (1) when all occupied cells contain mixed groups and minimum (0) when the objects of each group are all in different cells. The *SMI* between the three groups is also computed.

Table of distribution: The repartition of the objects (i.e. the number of objects from each group) in the different cells/sectors of the open field is presented in a table. The number of rows/columns is defined previously by the user (parameters dialog box).

Table of coordinates: The list of the coordinates of each point constituting the objects are presented in a table where the objects are in rows and the type of group is in columns. Group identity number, identity number of which aggregate the object is in, the object's width and length, and x and y coordinates in pixels for the three constitutive points (forward, gravity and backward) are included in this table.

Illustrative examples

Aggregation and dispersion indexes using artificial patterns

In order to validate the indexes computed by the software, we have designed artificial patterns using two groups of objects. The placement of each object has been chosen in order to reflect i) a high level of aggregation or no aggregation at all; ii) a high, medium, low or null level of inter-group affinity. The patterns are provided on the top three rows of Table 1 and illustrated by Figs 2.1–2.8. Additionally, ten replicates with random distribution of objects have been calculated. The last artificial patterns present the maxima and minima obtained in the random replicates (Table 1). The goal is to use a set of indexes able to discriminate between the different patterns.

Aggregation patterns can be discriminated by comparing the different indexes (see Table 1). For example, we will compare data for artificial patterns 1–4 (high aggregation) with 5–7 (high dispersion) for the red group, and between patterns 1–5 (high aggregation) and 6–7 (high dispersion) for the green group.

The three categorical values of aggregation (*% Aggr.*) reflect the overall aggregation pattern (100%, 50% or 0%) and for each group (100% or 0%) for extreme cases. The percentages of aggregation do not discriminate between two groups with a high level of aggregation, but their level of affinity differs.

The Aggregation Heterogeneity Index (*AHI*) can be used to discriminate between configurations where both groups are highly aggregative according to the presence or absence of affinity between them.

The Number of Aggregates index (*Nb Aggr.*) will show the exact number of aggregate but without providing affinity information (except in case of null affinity). When the two groups are not aggregating in the same way, the index is similar (for example

Table 1. Evolution of indexes in different virtual configurations. Specific patterns of intra-group aggregation and inter-group affinity based on eight virtual configurations composed of two groups of 8 objects (Red and Green). The last configuration (named Random) is the average of ten replicates of a random distribution of 16 objects and presents also minima and maxima of the indexes. Each configuration is illustrated by the Figures 2.1–2.8. Categories of indexes are: i) Inter-individual distances between all objects (*All*), or objects of the red (*Red*) or green groups (*Gr.*); ii) Nearest neighbours distances considering only the three nearest neighbours; iii) Number of aggregates (*Nb Aggr.*), Aggregation heterogenity index (*AHI*); Overall aggregation level (*%Aggr. All*) and aggregation level for each group (*%Aggr. Red*) and *%Aggr. Green*); iv) Spatial distribution index (*SDI*) for each group and Spatial Mixed Index (*SMI*) between both groups.

Groups:	**Configuration**	**Virtual 1**	**Virtual 2**	**Virtual 3**	**Virtual 4**	**Virtual 5**	**Virtual 6**	**Virtual 7**	**Virtual 8 (10 repl.)**	
Red	Aggregation	High	High	High	High	None	None	None	Random	
Green	Aggregation	High	High	High	High	High	None	None	Random	
Red/Green	Affinity	High	Medium	Low	None	None	Medium	None	Random	
Categories of index:									Minimum	Maximum
Inter-individual Distances	All <> All	62	59	61	454	364	326	363	301	374
	Red <> All	60	66	81	819	537	318	425	304	365
	Red <> Red	63	64	40	38	293	334	292	307	451
	Red <> Gr.	60	66	81	819	537	318	425	304	365
	Gr. <> All	60	66	81	819	537	318	425	304	365
	Gr. <> Gr.	66	39	38	39	37	335	293	254	400
Nearest Neighbours Distances	All <> All	37	39	38	149	160	195	223	177	247
	Red <> All	32	46	60	799	517	175	303	164	239
	Red <> Red	49	45	31	32	219	249	211	232	344
	Red <> Gr.	32	46	60	799	517	175	303	164	239
	Gr. <> All	31	51	62	799	410	169	307	162	210
	Gr. <> Gr.	49	32	31	31	31	257	220	169	320
Aggregation	Nb Aggr.	1	1	1	2	1	0	0	0	3
	AHI	1	1	1	0	0	0	0	0	1
	%Aggr. All	100	100	100	100	50	0	0	0	43.75
	%Aggr. Red	100	100	100	100	0	0	0	0	37.50
	%Aggr. Gr.	100	100	100	100	100	0	0	0	50.00
Spatial distrib.	SDI Red	0.5	0.25	0.13	0.13	1	1	1	0	1
	SDI Gr.	0.5	0.13	0.13	0.13	0.13	1	1	0	1
	SMI Red-Gr.	1	0	0	0	0	0	0	0	0.88

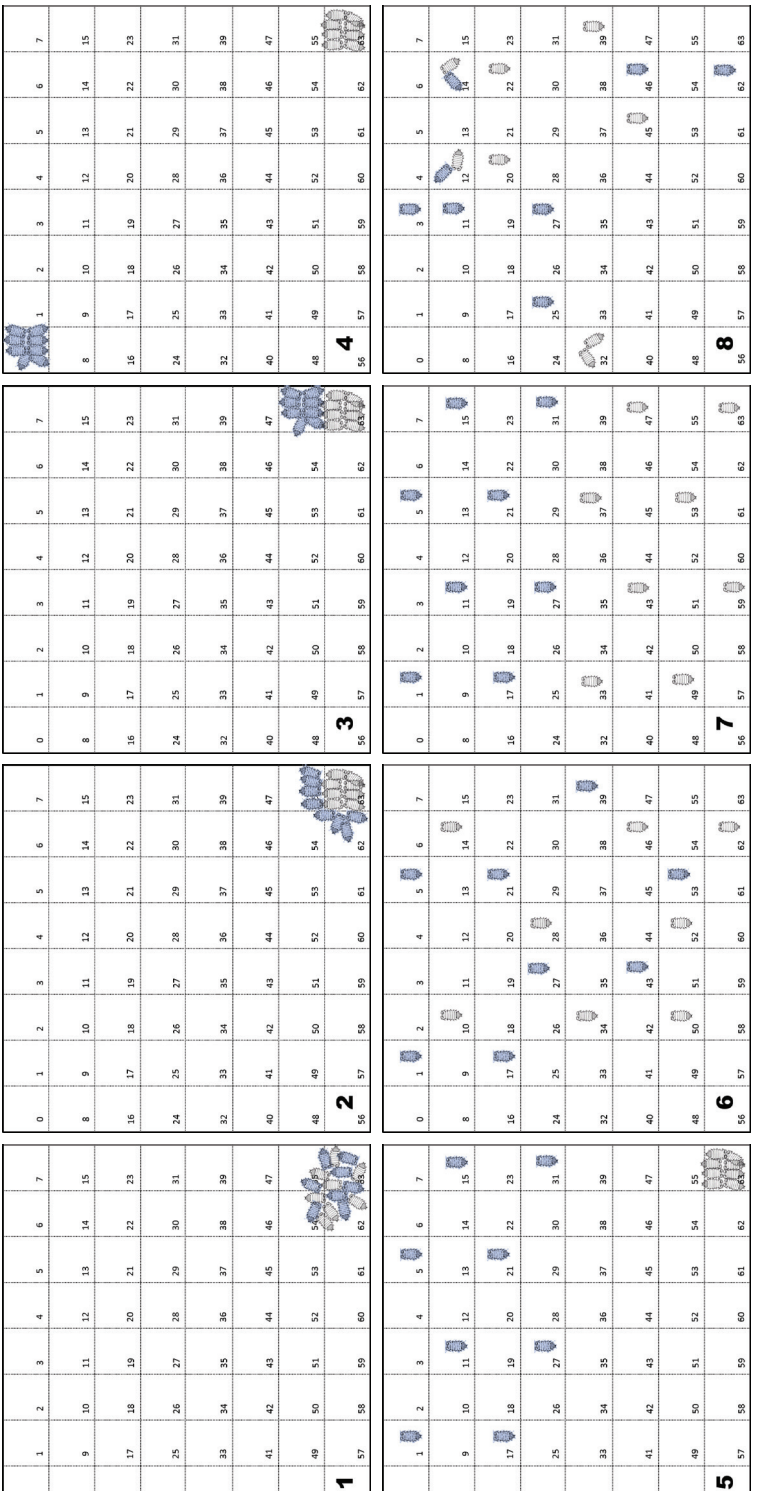

Figure 2. Virtual configurations used for software validation. Virtual configurations used to compile the data presented in the Table 1. Part 2.8 is one of the 10 replicates obtained with a random distribution. All other configurations have been designed in order to reach the desired level of aggregation and affinity between groups. The filled and empty shapes represented two virtual groups in the population.

between configurations 3 and 5). In our example group affinity will be characterised more using the spatial distribution index.

The Spatial Distribution Index (*SDI*) reflects the different aggregation patterns for both groups. However, in the case of high affinity between groups, the index increases significantly.

The other index of this category, the Spatial Mixed Index (*SMI*), reflects the high affinity in configuration 1. However, such an index is similar in the other configurations. These two indexes of spatial distribution are complementary to other indexes.

In the case of non-aggregative groups, showing or not a relative affinity (for example configuration 6 or 7 respectively), neither the percentage of aggregation nor the number of aggregates differs. In this case (see Fig. 2.6 and 2.7) the comparison of inter-individual distances will be the most informative to characterise individual affinity.

Inter-individual distance is a good indicator of aggregation level. Intra-group inter-individual distances are minimal when aggregation level is high. However, Table 1 shows that when affinity is high between groups, such intra-group inter-individual distances increase, as immediate neighbours can belong to both groups due to affinity (configurations 1–2). Use of distances to nearest neighbours limits this inconvenience. The inter-group distances are good indicators of the relationship between groups. Finally, the different levels of affinity between groups differentiate these two configurations.

Case study: Aggregation in Woodlice

The indexes have been tested using snapshots of individual distribution in terrestrial crustacean species (Oniscidea). Woodlice are good candidates for aggregation studies since such behaviour is widespread in this group and is explained as an adaptive response supporting their conquest of terrestrial life (Broly et al. 2013; Broly et al. 2012; Caubet et al. 1998; Caubet et al. 2008) and is under social component (Beauché and Richard 2013; Devigne et al. 2011). Such crustaceans present several specific constraints (weakness, group density. etc.) which lead to difficulties in real time tracking in comparison to insects.

First, we compared three gregarious species (groups): *Porcellio dilatatus* (PD), *Porcellio scaber* (PS) and *Cylisticus convexus* (CC). Three different combinations of two groups of eight individuals ("objects") are placed in a squared arena (width 12.3 cm) divided into 64 cells. After one hour, a snapshot is taken (Fig. 3) and the distribution of the individuals is analysed with NEIGHBOUR-IN. We used indexes in order to characterise our aggregates according to group characteristics. In a second step, we added the species *Armadillidium vulgare* (AV) to PD and PS. This species presents a more scattered aggregation pattern (Hassall et al. 2010). A snapshot is taken after one hour and analysed. The indexes are presented in Table 2 and the outputs concerning spatial distribution in Fig. 4 ("*Surfaces*" output display).

In our combinations, a first analysis focused on the main aggregate obtained in each combination using *AHI* and *SMI* indexes as descriptors of the quality of the ag-

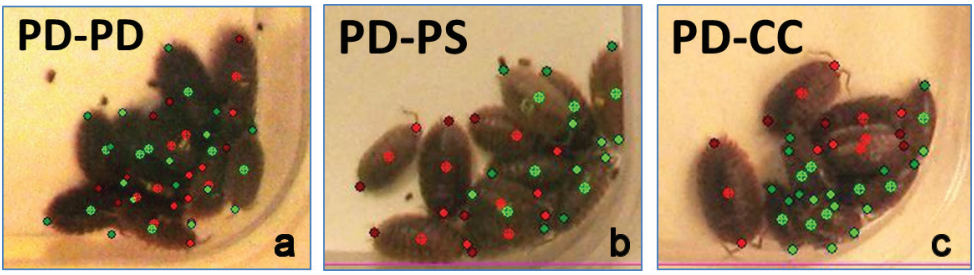

Figure 3. Aggregation heterogeneity in woodlice. Aggregation patterns of two groups of woodlice illustrating the Aggregation Heterogenity Index (*AHI*) and the Spatial Mixed Index (*SMI*). PD: *P. dilatatus,* PS: *P. scaber,* CC: *C. convexus*. Values of indexes: PD-PD: *AHI*=0.93 & *SMI*=0.80; PD-PS: *AHI*=0.67 & *SMI*=0.60; PD-CC: *AHI*=0.63 & *SMI*=0.33.

Table 2. Evolution of indexes in real configurations. Specific patterns of intra-group aggregation and inter-group affinity based on three real configurations composed of two groups of 8 woodlice (Red and Green). PD: *P. dilatatus*; PS: *P. scaber*; AV: *A. vulgare.* Each configuration is illustrated by figs 4.a-4.c. See Table 1 for the description of the categories of indexes.

Groups	Configuration	PD-PD	PD-PS	PD-AV
Species	Red	PD	PD	PD
	Green	PD	PS	AV
Categories of index:				
Inter-individual Distances	All <> All	188	174	541
	Red <> All	188	183	550
	Red <> Red	71	279	449
	Red <> Gr.	188	183	550
	Gr. <> All	188	183	550
	Gr. <> Gr.	305	50	614
Nearest Neighbours Distances	All <> All	113	107	302
	Red <> All	49	160	288
	Red <> Red	53	217	305
	Red <> Gr.	49	160	288
	Gr. <> All	155	54	321
	Gr. <> Gr.	130	37	492
Aggregation	Nb Aggr.	1	2	4
	AHI	0.93	0.67	0.20
	%Aggr. All	93.75	93.75	62.50
	%Aggr. Red	100.00	87.50	87.50
	%Aggr. Gr.	87.50	100.00	37.50
Spatial distrib.	SDI Red	0.500	0.500	0.75
	SDI Gr.	0.625	0.375	1
	SMI Red-Gr.	0.800	0.400	0.077

gregates (Fig. 3). In these snapshots the aggregate is composed of animals from both groups. However, the pattern of aggregation is different and the inter-individual distances cannot be used, since all individuals are close to one other. However, the use of

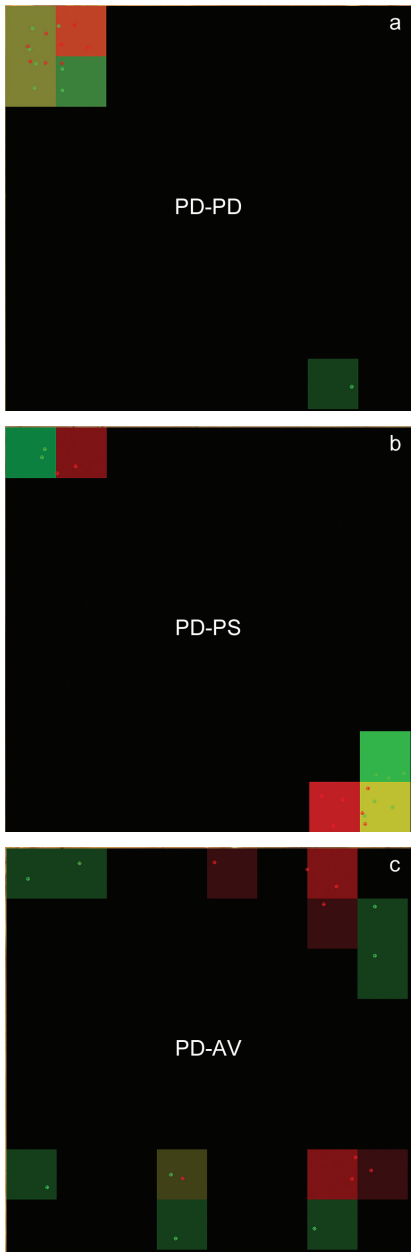

Figure 4. Spatial distribution in woodlice. Graphic outputs of spatial distribution patterns obtained in three configurations with monospecific or bispecific populations including two groups of eight individuals: **a** PD-PD: The two groups are *P. dilatatus* (red and green) **b** PD-PS: *P. dilatatus* (red) and *P. scaber* (green) **c** PD-AV: *P. dilatatus* (red) and *A. vulgare* (green). The outputs show 64 cells. Each cell is represented with a colour corresponding to the individual(s) in that cell. The colour is mixed using green and red proportional to the number of green and red individuals. If the cell is empty, the colour is black. The intensity of the colour reflects the number of individuals. The position of the individual is determined by its point G (centre-point).

AHI and *SMI* indexes can be informative since they differentiate the three combinations. When the two groups belong to the same species *P. dilatatus* (PD-PD, Fig. 3.a), affinity score is at a maximum between individuals and the *AHI* and *SMI* are at their highest score (respectively 0.93 and 0.80). When both groups belong to the species *P. dilatatus* and *Cylisticus convexus* (PD-CC, Fig. 3.c), the *AHI* and *SMI* are at their lowest (respectively 0.63 and 0.33). The intermediary configuration with the species *P. dilatatus* and *P. scaber* (PD-PS, Fig. 3.b) shows intermediary indexes (respectively 0.67 and 0.60). In conclusion, even in the case of very aggregative species, the quality of the aggregation pattern, matched with the affinity between individuals, can be characterised using a combination of complementary indexes.

The distribution of the two groups and the aggregation level appear to be very different according to the species pairing (Fig. 4). The indexes computed by NEIGHBOUR-IN reflect qualitative and quantitative differences in aggregation pattern variability well (see indexes on Table 2).

In the homospecific combination of the species *P. dilatatus* (PD-PD, Fig. 4.a) a single individual in the green group is isolated while all other individuals are crowded in a single mixed aggregate (five cells among 64 contain individuals). Only the cell containing the isolated individual is pure (bottom right corner on Fig. 4.a), while the four other cells contain animals that belong to both groups (*SMI* = 0.8; most of the cells contain individuals from both groups) and the distribution is totally mixed (*AHI* = 0.93; higher level of heterogeneity in the aggregate). The inter-individual distances show differences between red and green groups due to the isolated green individual (71 and 305 for reds and greens respectively). The nearest neighbour distances adjust the values especially for the green group (53 and 130 for reds and greens respectively). The intra- and inter-group distances are similar (53 and 49 pixels respectively). Aggregation indexes reflect the high level of aggregation of both groups (between 100% and 87.5% for reds and greens respectively). Concerning the spatial distribution index (*SDI*), the fact that one individual is isolated in the green group induces a small difference in the index (0.5 and 0.625 for red and green groups respectively).

In the heterospecific combination, with the species *P. dilatatus* – *P. scaber* (PD-PS, Fig. 4.b), we obtain two distinct aggregates located in the two opposed corners of the arena. Even if both species present a high level of aggregation (87.5% and 100% for PD and PS), the indexes are able to differentiate the quality of aggregation in comparison to the homospecific configuration (PD-PD). Both *AHI* and *SDI* values decrease (0.67 and 0.4 respectively). We observed that individuals in the same aggregate are sharing a single cell (Fig. 4.b). The other individuals are juxtaposed but not mixed, and stay close to conspecifics. Among the five occupied cells, four of them are occupied by individuals of the same group. Inter-individual distances increase because the aggregates are separated. The nearest neighbour distances adjust the values, and we observe an inter-group distance higher than in the homospecific configuration (169 rather than 49).

The third combination, using the two species *P. dilatatus* and *A. vulgare* (PD-AV, Fig. 4.c) presents another pattern of distribution: The green group (*A. vulgare*) appears

less aggregative than the red group (*P. dilatatus*) (37.5% and 87.5% respectively). In this configuration, four aggregates are identified by the software. The *AHI* index, reflecting the mixture of the aggregates, is very low (0.2) compared to homospecific and genera-related configurations (0.93 and 0.67 respectively). Moreover, the spatial distribution is completely different for both species: *A. vulgare* shows the maximum value of *SDI* index (1.0) meaning that each individual is in a different cell, and *P. dilatatus* shows a more dispersed distribution (0.75) than in the other two combinations (PD *vs*. PD and PD *vs*. PS) (0.5).

Discussion

Our image processing software, NEIGHBOUR-IN, provides indices with efficient discriminatory power to characterize and analyse group structure using individuals' coordinates. In the features of the software we integrate elementary statistical analysis and complementary index calculation that are important tools to describe aggregations, as well as a new index for more precise analysis. We provided examples based on random data and a case study using gregarious arthropods to highlight the accuracy of the output information. Moreover, the raw data, individual coordinates and location can be directly manipulated by the researcher for specific analysis such as simulation, modelling, and classic spatial statistics.

One of the assets of NEIGHBOUR-IN is the distinction between up to three groups and the open group size, which allow for a variety of applications. The differentiation between groups of individuals can be applied to compare intra-specific and inter-individual affinity according to size, age, sex, moult stage, health, and genetic relatedness at the individual level. Behavioural adaptive responses in inter-specific interactions is also an important field of investigation, and NEIGHBOUR-IN could be a new tool to study prey-predator, host-parasite, and commensalism impacts on group formation and composition. How the dispersion of animals in heterogeneous habitat and physical environment changes the aggregation pattern can also be investigated with this method. Moreover, the management of the image doesn't require a specific template or scale, and can be used with all type of images including aerial images of vertebrates, to macro photography of small invertebrates, and even picture under microscope with micro-organisms.

In comparison with the tools available in aggregation analysis, NEIGHBOUR-IN appears to be an accessible, light, and open solution. Our software is designed for analysis at a point-time however continuous monitoring of behavioural is not possible. The level of integration and data analysis complexity is smaller in comparison to GIS systems, which require user training and specific data templates and libraries. Other powerful softwares that track and analyse animal movement, such as Noldus ETHOVISION, analyse in real time and are not designed for snapshot analysis. Noldus ETHOVISION analyses are automated and qualitative information on aggregates could be missed. Many species present complex aggregate structures and often in three

dimensions so that a fully automatic tracking is necessarily imprecise. For example, when two individuals are superimposed, softwares like Noldus ETHOVISION lose track of the two individuals (one source of imprecision) and then randomly assign the initial characteristics to individuals when they separate, so that both intermediate and final results can lead to incoherency. Our semi-manual software allows manual localisation of individuals, increasing the precision of the spatial encoding, while keeping an automatic acquisition of results and analyses. Moreover, the user is able to identify orientation of individual (anterior and posterior extremities) with its perception area and consequently the possibility of interactions or not.

Finally, this software promises to evolve with new features, and could be used to generate and export distribution and coordinates database for other purposes. Potential fields of applications could be evolution of invasive species (animals and plants) distribution using aerial image, competition of fungus or microbial colonies, identification of harems in marine mammals grouping and so on.

The main limitations of NEIGHBOUR-IN software are in the potential excessive overlap of the individuals in an aggregate, and the total number of group and individuals taken into account. However, in both case, the user himself/herself is confronted to difficulties and the task, even if it is complicated, will be easier using NEIGHBOUR-IN. In comparison to a direct analysis of the image, the advantage to generating NEIGHBOUR-IN data files is that the image is saved with the coordinates but the statistics are managed separately, which allows the researcher to re-use the same files with further statistical analysis, as well as the integration of new graphic outputs and indexes.

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RESEARCH ARTICLE

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A fast GNU method to draw accurate scientific illustrations for taxonomy

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Abstract

Nowadays only digital figures are accepted by the most important journals of taxonomy. These may be produced by scanning conventional drawings, made with high precision technical ink-pens, which normally use capillary cartridge and various line widths. Digital drawing techniques that use vector graphics, have already been described in literature to support scientists in drawing figures and plates for scientific illustrations; these techniques use many different software and hardware devices. The present work gives step-by-step instructions on how to make accurate line drawings with a new procedure that uses bitmap graphics with the GNU Image Manipulation Program (GIMP). This method is noteworthy: it is very accurate, producing detailed lines at the highest resolution; the raster lines appear as realistic ink-made drawings; it is faster than the traditional way of making illustrations; everyone can use this simple technique; this method is completely free as it does not use expensive and licensed software and it can be used with different operating systems. The method has been developed drawing figures of terrestrial isopods and some examples are here given.

Keywords

Methodology, scientific illustrations, digital drawings, GIMP, Oniscidea

Introduction

Aiming to express a concept or convey a message, the use of a picture is certainly a clearer and understandable way compared to a text-only description. Images help reading a text and explain immediately what that text would represent. In biosystematics, descriptions of new plant and animal taxa are always combined with figures and plates in order to illustrate the anatomical parts and body details. Such figures are of great value for species identifications. Line drawings are normally used for many species descriptions. They can be produced by inexpensive means (ink and paper), as stated in the Council of Biology Editors' guide, *Illustrating Science*: "A good pen and ink drawing is pleasing, informative and reproduces well, even on poor grades of paper". The drawings should be organized to highlight the characters and their details. However, each figure should not be cluttered with too much detail, and there should be a pleasing balance between lines and white space (Winston 1999). When line drawing a body part, one usually makes it larger than requested by the size of the journal page in order to highlight details. The reduced final size will look sharper and small imperfections will usually be minimized (Zweifel 1961). Further notes on the preparation of illustrations for taxonomic papers are discussed in Mayr et al. (1953).

Taxonomists need images of good quality in describing taxa. As a rule, drawings are better detailed than stereo or light microscopes photographs since some details, which are often barely visible in a photograph, may be highlighted. When using photographs, focusing a complete detail requires many photos at a high level of magnification. Then, by using an appropriate software, it is afterwards possible to combine all the images, although the resulting quality is considerably low and require further processing steps.

Nowadays, only digital figures (drawings or photographs) are accepted by the most important taxonomic journals. The traditional way to generate high quality figures and plates for taxonomic papers, is to make pencil drawings first and then ink with high precision technical pens, which normally use capillary cartridge and different line widths. The single figures are then settled on a sheet of paper and each plate is scanned to a digital format. This method is sufficiently quick but may produce imperfect lines or gaps.

Digital drawing techniques that use vector graphics have already been described in literature to support scientists in drawing figures and plates for scientific illustrations (Coleman 2003, 2009); these techniques use a range of software packages and various hardware devices, such as digitiser boards or digital graphic pens.

The GNU Project (GNU is a recursive acronym meaning "GNU's not Unix") is a mass collaboration project of free software. Software that have been developed under the GNU Project guarantee these freedom-rights legally (via its license), and are therefore free software. GIMP is an acronym for GNU Image Manipulation Program. The GIMP is suitable for a variety of image manipulation tasks, including photo retouching, image construction and composition. GIMP can be used as a simple paint program, or as an expert quality photo retouching program, or again as an online batch processing system, a mass production image render, an image format converter, etc.

(GIMP documentation is available at: http://www.gimp.org/). GIMP is freely available from many sources for many operating systems. Most GNU/Linux distributions include the GIMP as a standard application. The GIMP software application is covered by the General Public License (GPL).

The purpose of this methodological work is to describe a new digital drawing method using GIMP software that helps the taxonomist in making high quality line drawings. This method was developed on drawings of terrestrial isopods for taxonomic papers. Some examples are here given.

Method

First of all GIMP has to be installed on a computer. Notes about installing GIMP on your computer are reported on the official GIMP website at the following URL: http://www.gimp.org/docs/. GIMP version 2.8.14 on a Mac computer (Mac Mini, late 2013, with Mac OS 10.10.1) has been used to illustrate the procedures of this paper.

The pencil drawings that need to be digital traced must be saved in a digital file or directly digitized in a Portable Document Format (a common PDF file).

Preparing the workspace

In order to prepare the window visualization at first access after the GIMP installation, all the panels that are shown should be closed. Only the "Toolbox" and the Dockable Dialogs "Layers" and "Tool Options" will be used and kept on screen:

Windows > Toolbox
Windows > Dockable Dialogs > Layers
Windows-> Dockable Dialogs > Tool Options.

Now the workspace should be arranged as reported in Fig. 1, with Image window (Fig. 1A) and three floating windows: Toolbox (Fig. 1B), Tool Options (Fig. 1C) and Layers (Fig. 1D). In the Toolbox select black and white for the Foreground and Background colours (Fig. 1E).

Using different versions of GIMP or different operative systems the toolbox and the two Dockable Dialogs are sometimes covered by the Image window and not visible as front windows. In GIMP for Windows OS you can change this setting from: Edit > Preferences > Window Management. In Mac OS from: GIMP > Preferences > Window Management (Fig. 2A). To have the toolbox and the other windows always on top of the screen change the Hint for the toolbox from 'Normal Window' to 'Utility Window'. You will be asked to restart GIMP in order to make changes effective.

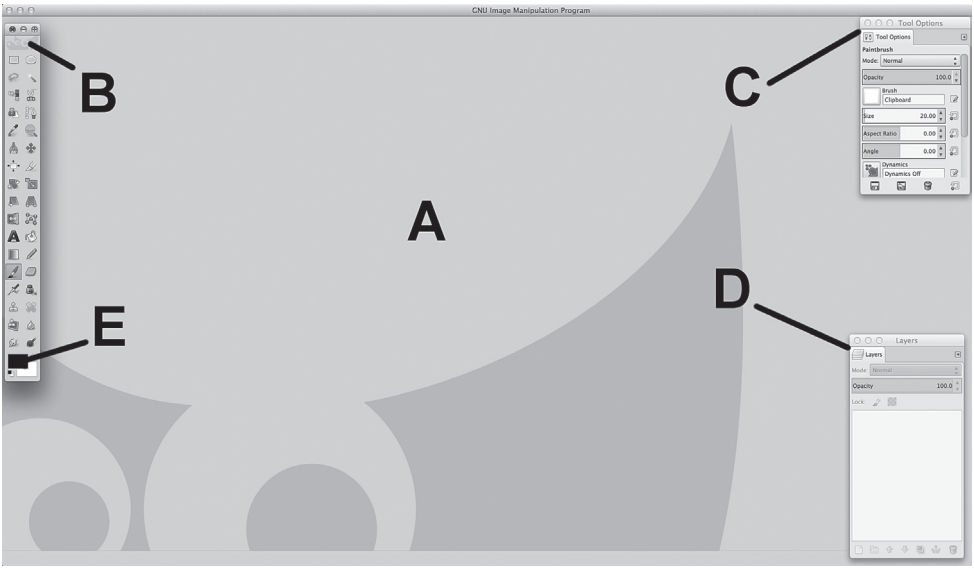

Figure 1. GNU Image Manipulation Program (GIMP, ver. 2.8.14) on a Mac OS. **A** Image window **B** Toolbox window **C** Tool Options window **D** Layers window **E** Foreground (black) and Background colours (white).

Step-by-step instructions

The user can follow the instructions of the present method according to two different levels of difficulty: beginner and advanced. Beginners are suggested to start testing the former method and then move to the advanced level once they have gained some experience.

Beginner level

1. Open the file that contains the pencil drawings: File > Open
   (with PDF files, set the resolution at 300 pixel/in) (Fig. 2B).
2. Create a new layer with a white background:
   Layer > New Layer
   (Layer Set "White" as Layer Fill Type) (Fig. 2C).
   Set the opacity of this new level to 60–80 in the Layers window.
3. Create another new layer for the first trace: Layer > New Layer
   (Set "Transparency" as Layer Fill Type).
4. Choose the brush left clicking on "Paintbrush Tool".
   On the "Tool Options" window, click on the square icon labelled "Brush" (Fig. 2D).
   Choose the brush No. 2. "Hardness 075".
   Set the "Size" to 3-5 points (Fig. 2E). Adjust the zoom level to 400–800%
5. Start drawing (see the "How to draw" section).
6. Once finished, set again the opacity of the "White" layer to 100, and the opacity of this first trace layer to 20–40, in order to better see the second trace.

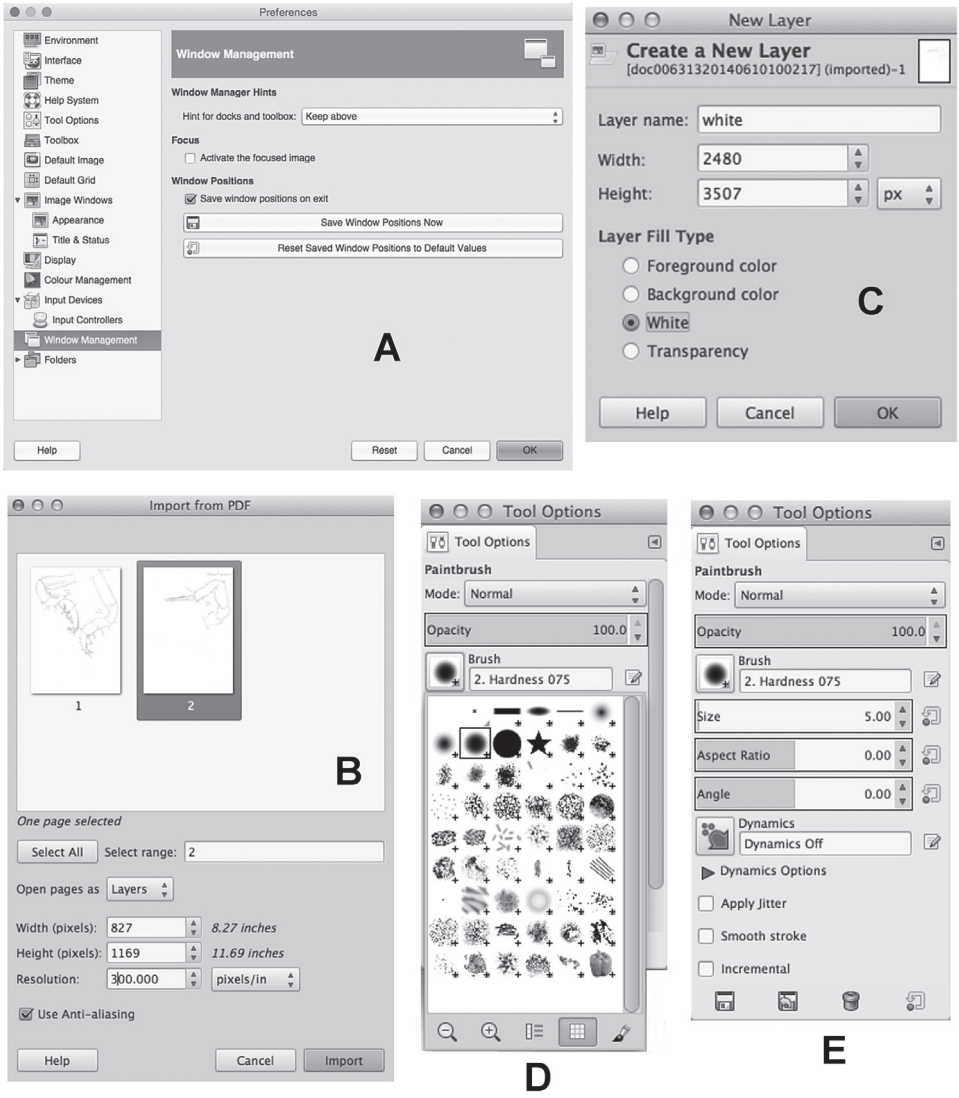

Figure 2. GNU Image Manipulation Program. **A** Preferences window, with the "Window Management" settings **B** "Import from PDF" window appears after a PDF file has been opened with the File > Open command of menu **C** New Layer window with the Layer Fill Type option set on "White" **D** Tool Options window of Brush Tool with the brush No. 2. Hardness 075, used in the present drawing method **E** Tool Options window (Brush Tool) with the brush size set on 5 pixels (5.00).

7. Create another new layer for the second trace: Layer > New Layer
   (Set "Transparency" as Layer Fill Type).
8. Start drawing in this new layer, following only the lines in the first trace. Remember to set the brush size to 5–7 points and to adjust the zoom level to 800–1100%.

Advanced level

1. Open the file that contains the pencil drawings: File > Open
   (with PDF files, set the resolution at 300 pixel/in)
2. Create a new layer for the trace: Layer > New Layer
   (Set "Transparency" as Layer Fill Type).
3. Choose the brush: click on "Paintbrush Tool". On the "Tool Options" window click on the square labelled "Brush". Choose the brush "2. Hardness 075". Set the "Size" to 5–6 points.
4. Start drawing (see the "How to draw" section), following the lines of pencil drawings.

How to draw (with a mouse)

- Set the zoom level to 400–800% for the first trace, 800–1100% for the second (or at advanced level);
- choose an appropriate starting point (e.g. a corner or an intersection);
- choose the brush tool with the right size (see above) and do a first click with the left button (Fig. 3C) at the starting point you have chosen;
- start to trace out the pencil line by pressing the shift key on the keyboard (Fig. 3A), then move the mouse pointer along the pencil line: a guide line will appear to help you to trace a segment (Fig. 3B);
- stop the pointer where you consider worthwhile; left click once again to trace a segment;
- press the space bar of the keyboard (Fig. 3A) to move along the drawing, only moving the mouse, without clicking any other button;
- change the brush size whenever you need to draw smaller details;
- find a suitable balance between several closer points or less numerous but more distant points (i.e. the suitable balance between the time you have and the quality of your drawings).

Shorter segments will appear as a continuous line and the quality will be even better in the printed version since the figure size is smaller than the digital image.

Drawing dashed lines

- Choose the "Path tool" in the toolbox (Fig. 4A);
- left click several times following the pencil line;
- once finished, click "Stroke path" in the "Tool options" dialog window (Fig. 4B);
- in the "Stroke path" window (Fig. 4C), choose the line width (e.g. 4 or 5 pixels) and open the "Line Style" dialog;

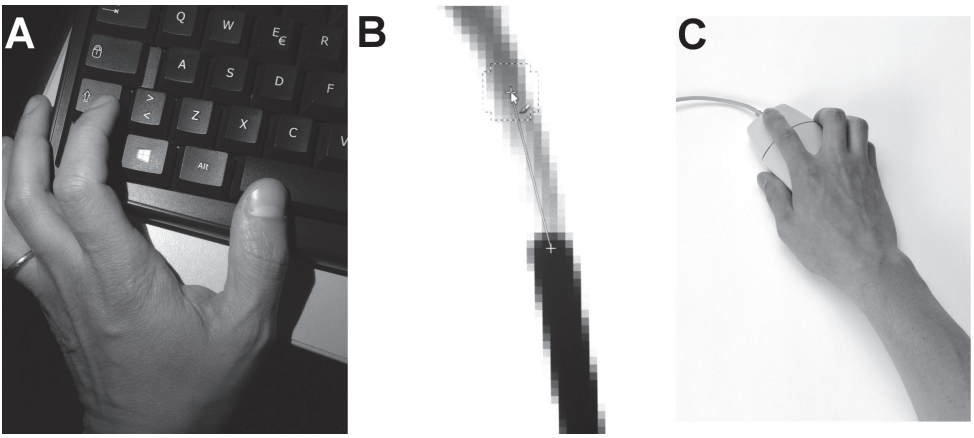

Figure 3. How to draw lines with GNU Image Manipulation Program. **A** Left hand position: with Shift key pressed to draw little segments, space bar to move the screen visual along the drawing **B** Portion of the Image window (at 800% zoom level) showing the drawing guide-line **C** Right hand position with a common mouse.

– choose "round" (the second icon) for "Cap style" in the "Line style" option, then "Medium dashed" in the "Dash preset" option (Fig. 4C);
– click on the "Stroke" button and then again on the "Paintbrush tool" to see the result (Fig. 4D).

Sorting out plates

First of all open a new file with a blank image. Choose File > New and select A4 (300 ppi) in the Template menu, then click "OK" (Fig. 5A). The size of this new image will be 2480 × 3508 pixels at 300 points per inches. It is possible to change the resolution, e.g. at 600 ppi, opening the Advanced Options (Fig. 5A)

In order to move the trace that has been already drawn into the new plate: open the .xcf file and select the layer that contains that trace. Use the 'Free Select Tool' to carve out the part of the drawing; left click several times following the trace outline. Then copy the selection (Edit > Copy) and paste it (Edit > Paste) into the new blank image. A new layer, named 'Floating Selection' will appear in the Layers Dialog (Fig. 5B); right click on this new layer and choose 'To a new layer' to create a new layer (named "Pasted Layer") with the clipping (Fig. 5C). This is particularly useful in order to rearrange the plate later. It is also possible to move the entire layer (with a simple drag and drop of the layer into the new image) but this step will increase the file size. The new layer can be renamed by double-clicking on its name in the "Layers" window.

Next step is to move, scale, flip or rotate the drawings to set them in the right position. In the toolbox it is possible to find the right instruments (Fig. 5D).

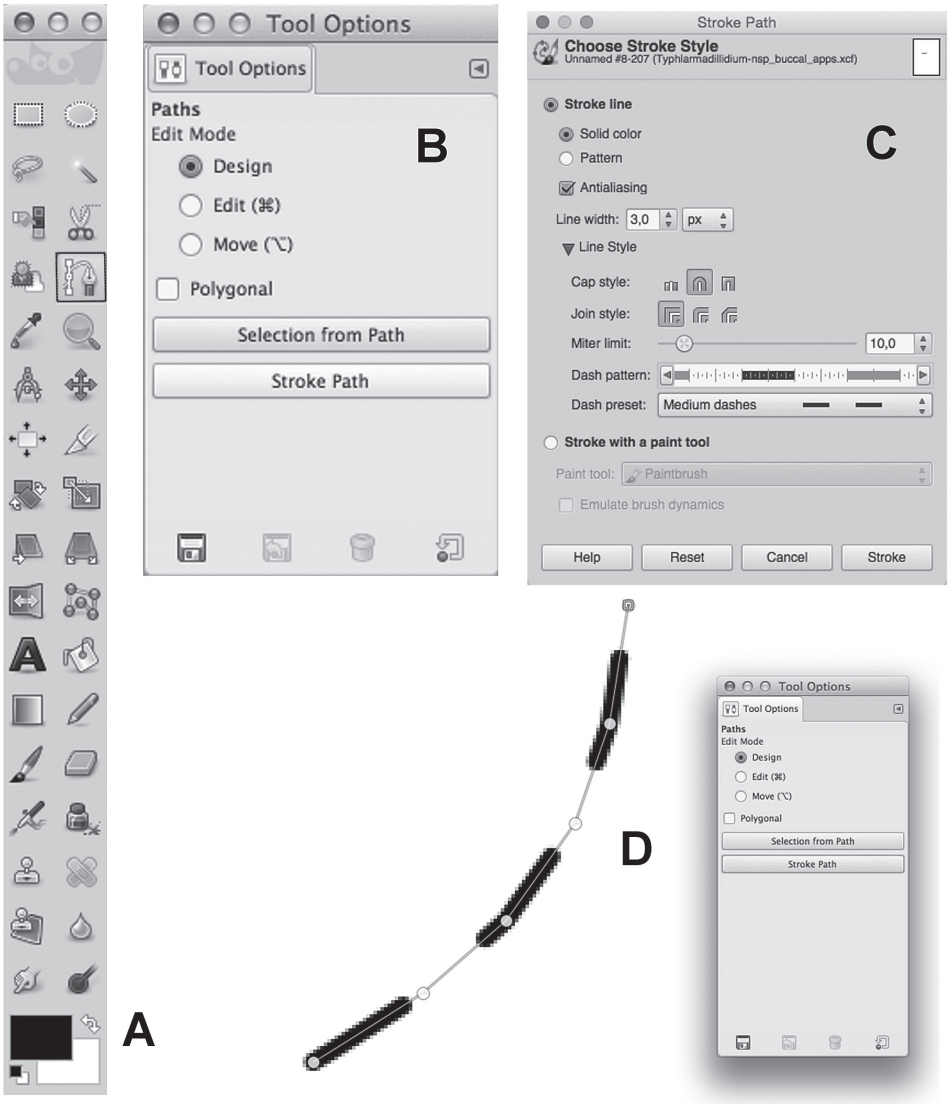

Figure 4. GNU Image Manipulation Program. **A** Toolbox window: Path Tool is marked with a black square **B** Tool Options window of Path Tool **C** Stroke Path windows with the settings for a "Medium dashed" line (3 px) **D** Portion of the Image window showing the result (at 400% zoom level) of the "Stroke Path" button.

Move. Use the "Move Tool" in the Toolbox. In the "Layers" window select the layer with the trace. In the Tool Option Window, choose "Layer" (the first icon) in the "Move" option and "Move the active layer" in the "Tool Toggle" option. Then, it is possible to move the trace in the right position by left clicking on the trace and dragging the layer.

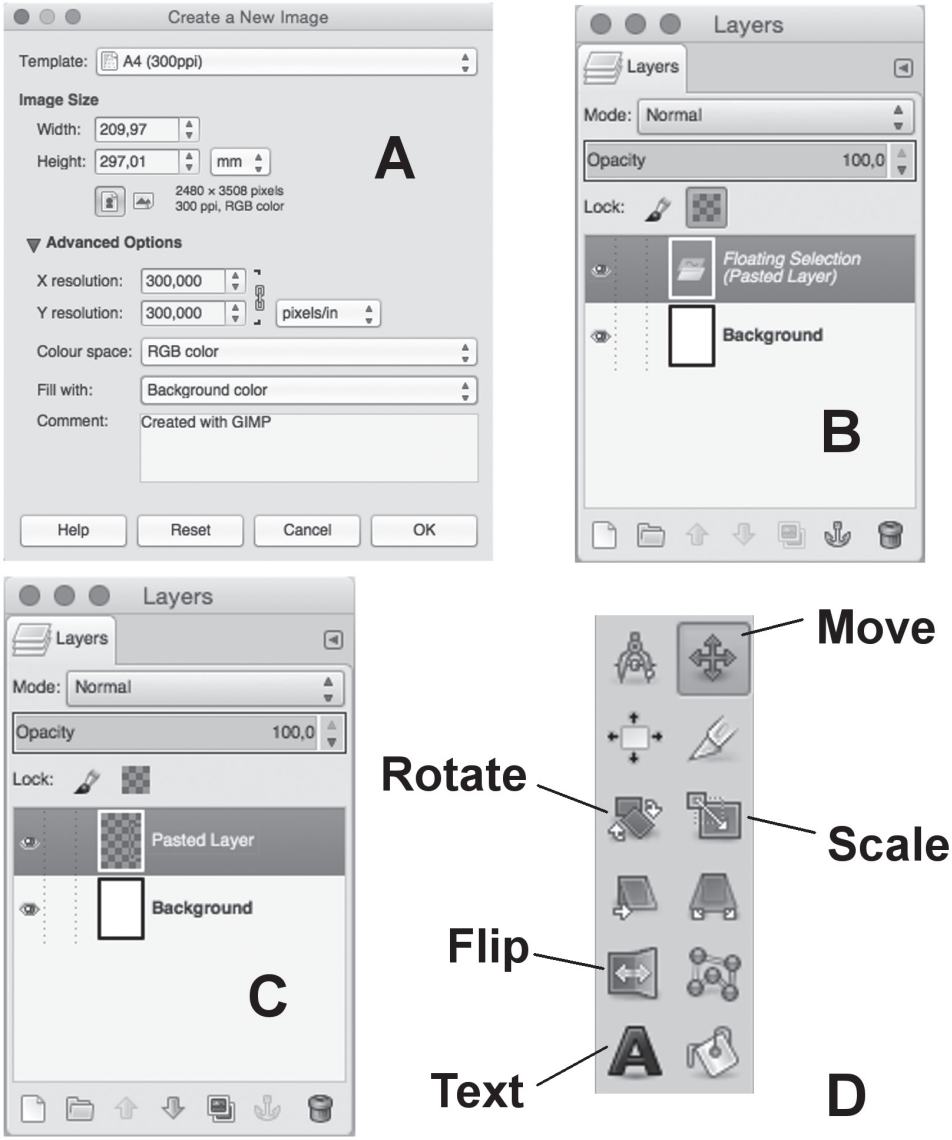

Figure 5. GNU Image Manipulation Program. **A** "Create a New Image" window with settings for a blank page in international A4 format **B** Layers window after a trace was pasted into a new image, the "Floating Selection" layer is showed **C** "Pasted Layer" on the same window after the "Floating Selection" was transformed in a new layer **D** Portion of the Toolbox window showing the tool icons (explanation in the text).

Scale. Use the "Scale Tool" in the Toolbox. In the Tool Option Window, choose "Layer" in the "Transform" option and leave the other options with the default values. Left click on the trace and a grid and a "Scale" window will appear helping the user to adjust the image at the right size. It is possible to use percentages by changing the "pixels" option.

Flip. Similarly, use the "Flip Tool". In the Tool Option Window, choose "Layer" in the "Affect" option and then choose "Horizontal" or "Vertical" in the "Flip type" option. Left click on the trace and the drawing will be mirrored upside down or left/right.

Rotate. It is also possible to rotate a drawing with the "Rotate Tool". As before, left click on the trace and a grid and a "Scale" window will appear helping the user to adjust the image at the right angle. Then left click on the "Rotate" button to set the new arrangement.

Once completed, it is necessary to repeat the steps above to insert all the drawings into the new plate.

Lettering. Easily, it is possible to add text (e.g. numbers and letters) on the new plate using the "Text Tool". A common option in the "Tool Options" window is: "Arial" in the Font box and 80–100 in the Size box. A new layer will appear with the text. It can be easily moved with the "Move Tool" just as explained before.

It is also possible to crop the image in order to eliminate the blank space around the traces.

This can be easily done with the "Crop Tool" in the Toolbox. Left-click and drag the mouse pointer to crop the part of the plate.

Saving the files

Once completed it is important to save the work in different files. Precisely, the user should be save the draft files in the GIMP proprietary format XCF (eXperimental Computing Facility) in order to leave the possibility to work again on the different layers. In the "File" menu, choose "Save" (or "Save As"); then the user will be asked to add a file name and select a destination folder. Otherwise, it is possible to save the plates as one-layer files in a high resolution format, such as TIFF files, normally used for publication in a scientific journal. This step should be done with the "Export" (or "Export As") option of the "File" menu. A new dialog window will appear on the screen: in this case choose "TIFF image" in the "Select File Type" option (Fig. 6A). Then, it is possible to set the level of compression (that is the quality of images). Generally a LZW compression offer the right balance between quality of the printed image and an appropriate file size. This option is specifically requested by several journals (Fig. 6B).

Results

The method here explained, produce three different kind of files: the original digitized pencil drawings (normally PDF or TIFF files), the .xcf files of the traces, the .xcf files of the plates. These last ones are used to export the plates in 'Tagged Image File Format' (TIF) that may be directly uploaded to the journal submission website. All those files should be kept because they could be useful in the future for many purposes.

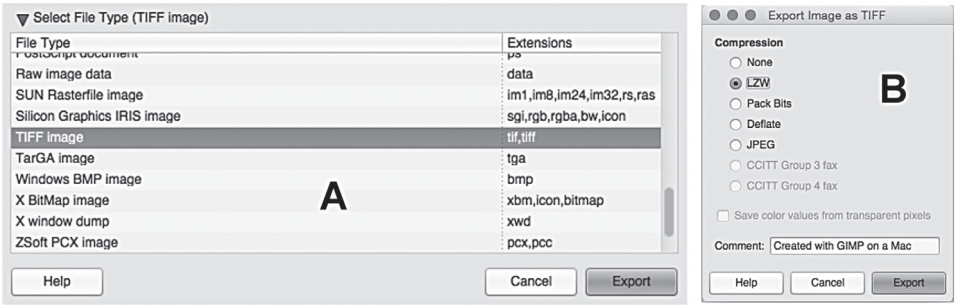

Figure 6. GNU Image Manipulation Program. **A** Portion of the "Export Image" window, showing the "Select File Type" menu with the settings for a TIFF image **B** "Export Image as TIFF" window, with the settings for LZW compression.

The final result is to have perfect lines without any signs of tremble. The black lines appear as a sharp continuous line in the final version, because the size of the printed image is not as big as the digital image. Generally, the use of digital drawing techniques allows undoing the last action and trying again after an error, until the result is satisfying.

Some illustrations made with the method of the present paper, were already published in other articles about terrestrial isopods: figure 1 in Medini-Bouaziz et al. (2006); figure 2 in Montesanto et al. (2007); figures 2, 3 in Montesanto et al. (2008); figures 1–5 in Montesanto et al. (2011); figures 1, 2 in Messina et al. (2011); figure 1 in Messina et al. (2012); figures 6, 7, and 9 in Montesanto et al. (2012); figure 1 in Lupetti et al. (2013); figures 2, 3 in Montesanto et al. (2013); figures 1–6 in Messina et al. (2014). Some indications about drawing terrestrial isopods for taxomomic papers are reported in Figure 7, whereas magnifications show the differences of the brush size for different anatomical parts.

Finally, due to the practice made with the method here introduced, some other suggestion are proposed to the reader: draw the possible dotted shadows on a new layer above the other ones; use maximum zoom level for details, even more than 1100%; delete errors with the Eraser Tool (in the toolbox) or by clicking "Undo" in the Edit menu; always look at the general shape of the curve (reducing zoom level); avoid excessively long segments in order to point out no edges; try different size of brushes for setae, spines and other little details (see Fig. 7).

Discussion

In a work of biological taxonomy, the part that requires more time, is the preparation of illustrations. There are always many species that need to be described so some taxonomists usually describe more species, not so detailed, others describe fewer species, but very detailed (Coleman 2006). Our predecessors and contemporary isopodologists

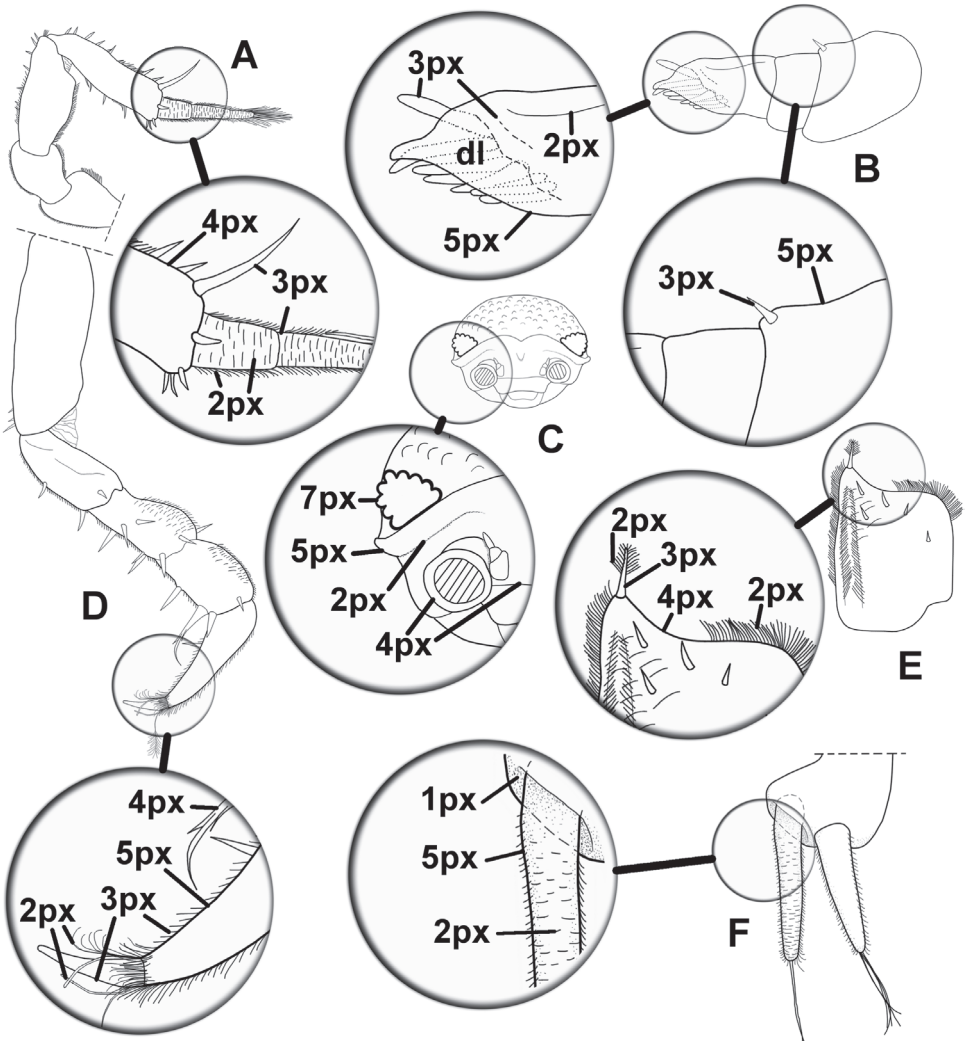

Figure 7. Examples of line drawings of terrestrial isopods anatomical parts. Magnifications in the black circles indicate the brush size (see also the "How to draw" section). **A** Antenna **B** Antennula **C** Cephalon (front view) **D** Pereopod 1 **E** Exopod of pleopod 4 **F** Uropod; dl, dotted lines (Commands: Paths Tool > Stroke Path in the Tool Options > Line style, Dash preset: Dense dots).

published exemplary literature (e.g. Vandel 1960, 1962; Caruso and Lombardo 1978, 1982; Schmalfuss 1996; Taiti and Ferrara 1996; Schmidt 2002, 2003, 2007; Taiti and Argano 2009; Taiti and Gruber 2010); every taxonomist would aim to publish such high quality works that would be still useful in the future, but the limited time is always a problem. Software packages like DELTA (Dallwitz et al. 1993; Dallwitz et al. 1998), can automatically generate dichotomous keys and also taxonomical diagnoses and descriptions, that accelerate the description process. Nevertheless, making illustrations for taxonomy papers and also for software like DELTA, is always a long process

(Coleman 2006). At first a pencil drawing is made often using a camera lucida; then these drawings are traced again on plates for the publication.

Other similar methods of digital drawings techniques, but with the use of vector graphics, have already been described in literature (Bouck and Thistle 1999, Coleman 2003, Bober and Riehl 2014). Such methods were a significant step forward in speeding up the time consuming part of a taxonomic description, but they nevertheless showed some weak spots. Actually, those techniques require the use of several software and various hardware devices (such as digitiser boards and digital graphic pens), and plus the cost of complicate and very expensive software (e.g. Adobe Illustrator™, Adobe Photoshop™). Nonetheless, simple and free vector graphics software (e.g. Inkscape, https://inkscape.org) may be used in order to obtain good results.

On the other hand, the method here discussed produces good results in terms of image quality and precision, saving time and costs. Specific strengths are listed here: 1. It is simple but accurate, producing detailed lines at the highest resolution. 2. Small structures can be greatly magnified, so that drawing is easier than using common inking on paper. 3. The raster lines appear as realistic ink-made drawings. 4. It is much faster than the traditional way of making illustrations. 5. Everyone can learn to use this simple technique; it can also be used by technical staff or even inexperienced volunteers. 6. This method is completely free as it doesn't use expensive and licensed software. 7. As reported in the introduction of this article, GIMP is available for different platforms (Windows, Mac OS, or Linux), thus the work files can be moved in different computers maintaining the same file extension (.xcf).

Genuinely, some specialist can find the last points debatable preferring, therefore, the use of vector graphics. For example, only vector graphics allows to scale a drawing supporting the maximum detail; on the other hand when bitmap line drawings are magnified over 200%, they clearly show a typical 'pixels' vision. In addition, some other specialist can find a better choice working with a drawing board instead of a simple mouse, and this is also possible with GIMP. These are clearly subjective points. The best suggestion is try as many methods as possible. Then, once practiced, the users will have their better choice.

The basis for the digital drawing method here proposed is a conventional pencil drawing, made with a microscope and a camera lucida. However, it is possible to use the same method starting with a work of stack microphotography, in order to avoid the time-taking drawing process. For this purpose many software are available to combine images of different depth of focus into one photo, such as Auto-Montage (http://www.syncroscopy.com/auto-montage/), CombineZP (http://www.hadleyweb.pwp.blueyonder.co.uk/), Helicon Focus (http://www.heliconsoft.com/), Zerene Stacker (http://www.zerenesystems.com/). Recently, an interesting comparison of the focus stacking software packages has been published by Brecko et al. (2014), as a possible solution for mass digitization of type specimens; an ant of the genus *Meranoplus* and a beetle of the genus *Trachys* were tested. Nonetheless, these photographic methods show some issues (see also Coleman 2006). Actually, a drawing can show important characters that are difficult to see in a photo. Even small details, such as aestetaschs of

antennulae, so important for terrestrial isopods taxonomy, can be easily shown, e.g. in Trichoniscidae, fine lines between articles of antennal flagellum, or other characters that are often used for species taxonomy. A detailed illustration may be considered as an interpretation, it is not only a simple description of the morphology. So, it is possible to point out some structures that are covered by others (Coleman 2006). Generally, one other big advantage of such digital inking methods is that technical assistants or other volunteers may be employed in order to save time. The specialist can afterwards correct the illustrations, if indispensable (see also Coleman 2003, 2006).

GIMP offers many other opportunities that have not been described in this paper. In fact, the use of GIMP has been cited in other papers on digital drawing (such as: Sidorchuk and Vorontsov 2014). Here have been reported only the essential informations needed to obtain high-quality figures and plates which are suitable for online and printed publication. Any further request for clarification may be asked to the author.

Additional material

An explanatory playlist (with 6 videos) has been published at the following URL, in order to facilitate the users with the method here showed:

https://www.youtube.com/playlist?list=PLHuMNpWqA6OxGAgzk6yp07a55KV7i2caJ

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