Advances in Terrestrial Isopod Biology

Edited by

Jasna Štrus, Stefano Taiti, Spyros Sfenthourakis

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On the front cover: *Titanethes albus* (Koch, 1841). Photo by Helmut Schmalfuss.

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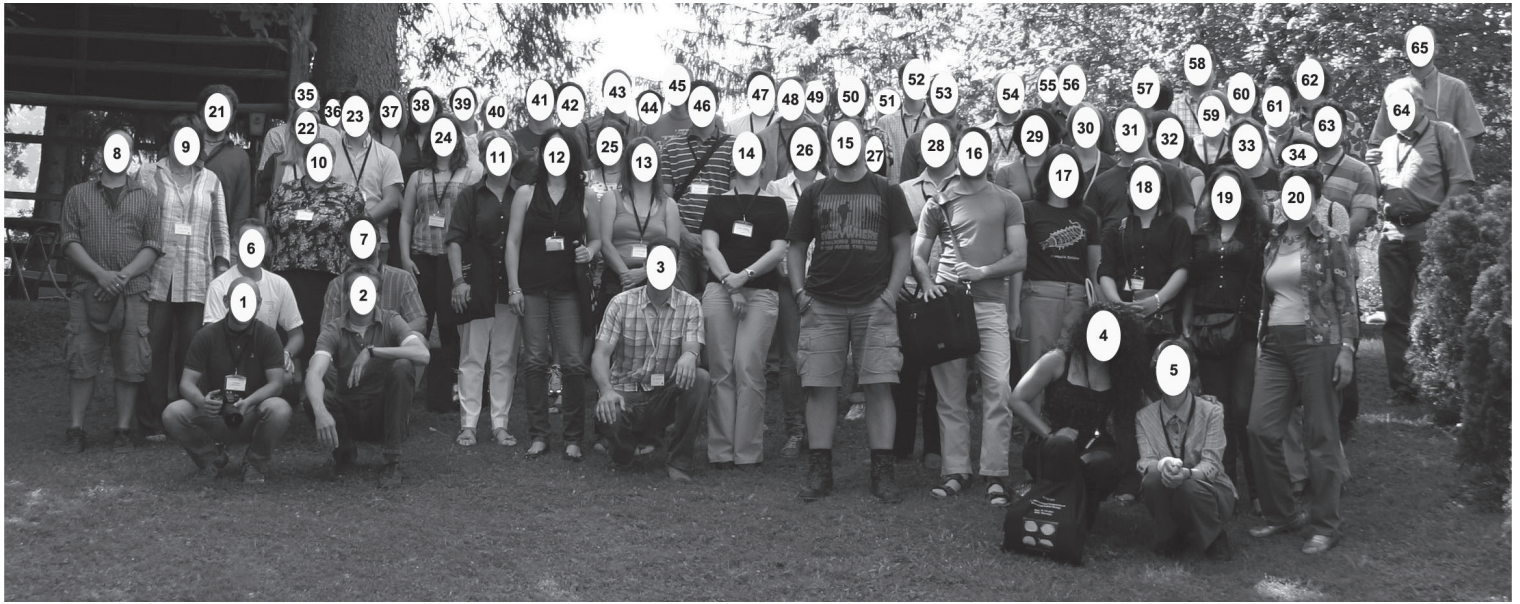

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Preface

> Nature is beautiful and science is exciting. The beauty of nature lies in detail and the message of science in generality. Exciting principles are best illustrated by well-chosen particulars.
>
> (adapted from S.J.Gould, Wonderful Life)

Curiosity drives research and biologists explore life; among them a group of enthusiastic people is interested in lifestyles of woodlice. Terrestrial isopods are unique crustaceans inhabiting different habitats worldwide: from seashores to deserts and caves, as well as human homes. They form a community of decomposers together with bacteria, fungi and other animals, transforming organic matter to soil and other substrates.

For the last thirty years, woodlice fans have met on different occasions to exchange their ideas and research results on woodlice biology. A survey of the seven symposia on the Biology of Terrestrial isopods, held between 1983 and 2007, was presented at the eighth International Symposium on Terrestrial Isopod Biology (ISTIB2011) by Helmut Schmalfuss with the following words:

"Photographs and anecdotes are intended to give a personal impression to show that we are a rather normal group of human beings who have chosen a somewhat extravagant group of organisms to work with."

The first meeting on the biology of terrestrial isopods was held in London in 1983, organized by S. L. Sutton and D. M. Holdich, following a suggestion of M. R. Warburg, and sponsored by the Zoological Society of London. The second meeting was held in Urbino, Italy in 1986, organized by R. Argano, P. Del Grande, F. Ferrara, C. Manicastri, H. Schmalfuss and S. Taiti under the auspices of the Consiglio Nazionale delle Ricerche and the Unione Zoologica Italiana. The third meeting followed in 1990 in Poitiers, France and was organized by P. Juchault and P. Mocquard from Laboratoire de Biologie Animale de l'Université de Poitiers. Almost 70 researchers and students shared the joy of seeing live woodlice displayed in the auditorium. The fourth meeting was organized by M. R. Warburg and E. Hornung in Technion, Haifa in 1997. In 2001 the fifth symposium was held in Irakleion, Greece, hosted by the Natural History Museum of Crete and organized by S. Sfenthourakis. The highlights of the meeting were field trips with enjoyable Greek landscape and woodlice diversity. The sixth meeting in 2004, with dynamic exchange of ideas and views on woodlice biology and ecotoxicology, was hosted by the University of Aveiro, Portugal, organized by S. Loureiro and A. Soares. In Tunis in 2007, the seventh meeting was hosted by the University of Tunis and organized by F. Charfi-Cheikhrouha, M. S. Achouri, S. Hamaied and K. Nasri-Ammar. We saw a mosaic of woodlice research from different countries. The eighth meeting ISTIB2011 in Bled, Slovenia, organized by J. Štrus and P. Zidar, brought together 77 participants contributing to the knowledge on woodlice biology. We exchanged ideas and experience in the fields of systematics and biogeog-

raphy, morphology and physiology, mineralized organic matrices, molecular biology and microbiology, evolutionary and developmental biology, ecology and ecotoxicology, woodlice and agro-systems.

The present volume includes 20 articles out of 68 oral and poster communications presented at ISTIB2011, covering a large number of topics. In particular, new data on isopod distribution from regions that have not been well studied, such as the former USSR and Iran, are presented by Gongalsky & Kuznetsova and Kashani & Sari, respectively. Developmental aspects, focused on the crucial process of molting, are explored by Vittori et al., Žnidaršič et al. and Mrak et al., while Ziegler & Seidl provide insights into tools useful for such -and other- studies of the cuticle. Montesanto et al. combine developmental studies with new data on reproductive biology of a local endemic species. Another aspect pertaining to developmental biology is presented by Luquet, who makes a review of biomineralization in crustaceans. The exciting topic of *Wolbachia* infections has been an important part of such symposia for a long time, and in this volume it is reviewed for isopods and related taxa by Cordeaux et al. Behavioral studies on terrestrial isopods have not attracted scientific attention to the degree they should have, as demostrated by the intriguing studies of Broly et al., Drahokoupilová & Tuf and Quadros et al., who explore a diverse set of behavioral issues, such as aggregation, visual signals and tonic immobility. Community ecology has always benefited from studies on Oniscidea assemblages, as shown here by Antonović et al., Tajovsky et al., Vilisics et al. and Messina et al., who offer valuable information that can also prove useful for conservation and urban ecology. The important ecological role of terrestrial isopods as decomposers renders them perfect models for studies on soil ecology and ecotoxicological approaches. These issues are covered here by the work of Wood et al., and Vilisics et al. on feeding rates and litter decomposition, as well as by Novak et al. on toxic effects of feeding with TiO_2. The general background and state of the art for such ecotoxicological studies are reviewed by van Gestel.

In the last thirty years biology has undergone significant changes, discovering life in its different forms, deciphering genomes of many species, searching answers to environmental and climatic issues, and educating people on how to conserve nature. Woodlice have not changed during this period, they are still here to be explored, described and used as experimental animals in laboratories worldwide, with a common goal: to keep life on the planet pleasant and sustainable.

We would like to thank here all the colleagues who have dedicated some of their precious time in revising the manuscripts included in this volume, thus contributing to their quality, as well as Dr. Lyubomir Penev and Ivailo Stoyanov of Pensoft Publishers who enthusiastically encouraged and supported this publication.

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RESEARCH ARTICLE

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Cartographic analysis of woodlice fauna of the former USSR

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Abstract

An inventory of the woodlice fauna of the former USSR yielded 190 species, 64 of them were recorded from the territory of Russia. According to the cartographic analysis, the limits of distribution of epigean terrestrial isopods over the area, excluding mountains, is explained by temperature. No woodlice records were found outside the isocline of 120 days a year with the mean daily air temperature >10°C. The highest species diversity was found between the isoclines of 180 and 210 days. These areas correspond to forest-steppe and steppe zones.

Keywords

Woodlice, mean annual air temperature, database, Russia

Introduction

Studies of spatial differentiation of various taxa are among the most important frontiers of modern biogeography. For some well-studied groups, mainly, vertebrates and plants, such trends are already discovered (Loiselle et al. 2003; Guisan and Thuiller 2005; Grenouillet et al. 2011), but for soil-dwelling invertebrates they are only at the stage of species inventory. However, there are certain groups of invertebrates for which analysis of spatial differentiation is already possible due to the large number of records from different geographical localities. Woodlice are among such groups.

There is no faunistic list of terrestrial isopods for the territory of the former USSR until now, as well as of the territory of Russia. However, there are extensive regional lists (Borutzky 1948, 1953; Zalesskaya and Rybalov 1982; Khisametdinova 2007;

Gongalsky and Kuznetsova 2011), and numerous records scattered in the literature devoted to soil macrofauna. At the same time, there are only a few ecological studies about factors affecting woodlice distribution over regions of the former USSR (Gongalsky et al. 2005; Khisametdinova 2009).

The aim of the study is to determine the factors affecting woodlice distribution over the plain area of the former Soviet Union. To achieve this, an inventory of species distribution across the study area was made. The task was to create a database indicating locations with woodlice presence/absence overlaid with several environmental variable values distribution.

Material and methods

Database

The first step was to compile a list of species for the study area. We made a database of isopod presence or absence in the locations across the whole territory of the former USSR (both plains and mountains). For each record the database includes information about date, data source, geographical coordinates, location, isopod species list or information about woodlice absence in the soil fauna list, biotope, and natural zone.

Three types of information sources of terrestrial isopod locations were used: i) available literature on soil fauna surveys; ii) collections of the Zoological Museum of Moscow State University (Moscow, Russia) and the Zoological Institute of the Russian Academy of Sciences (St.-Petersburg, Russia); and iii) authors' personal collections. Here we provide a list of woodlice from the territory of the former USSR since some species and localities were not included in the list of Schmalfuss (2003), although it covered the majority of species. To work with regional databases, a specific list would be useful. Since such a list for this area did not exist, the proposed compilation would be a start to be completed in the future. We used the taxonomic system proposed by Schmalfuss (2003) for species naming. Isopod absence was recorded only in extensively surveyed locations.

For cartographic analysis, 259 locations were chosen, 44 of which with woodlice absence. Due to the difficulty of tracing ecological trends in the mountains, only plain territories were involved into the analysis. Some species were excluded from the analysis: i) synanthropic species and ii) species inhabiting azonal locations, such as sea coasts, caves and anthills.

Then database records with isopod presence or absence locations were laid on the geographic maps to perform cartographic analysis.

Cartographic analysis

The map of woodlice distribution was visually compared with the maps of environmental factors (mean annual temperature; the period with temperature above 10°C;

mean precipitation; permafrost distribution; soil pH and soil type; vegetation type; natural zones) found in the Agricultural Atlas of the USSR (Tulupnikov 1960) and the Geographical Atlas of the USSR (Kolosova 1980). The data were verified using the WorldClim database (Hijmans et al. 2005).

The database is maintained in MS Excel. Cartographic analysis is done in MapInfo 8.5.

Results and discussion

Limits of isopod distribution

Woodlice have not been recorded northwards the isocline of 120 days a year with temperature >10°C (Fig. 1). The northern border of woodlice distribution matches the distribution of this parameter. Other parameters did not coincide with isopod distribution as well as with this isocline (data not shown).

Species diversity

In total, 190 species were recorded from the territory of the former USSR (Appendix 1). Among them, 64 were recorded from the territory of Russia. Northernmost natural

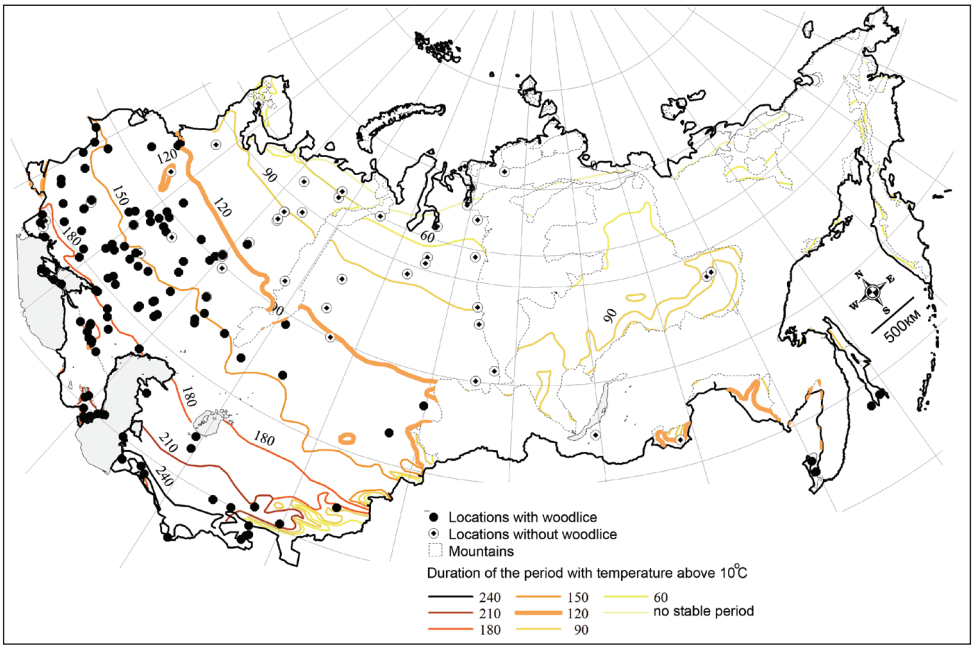

Figure 1. Map of woodlice presence or absence over the plain territory of the former USSR. The duration of period with temperature >10°C is adapted from Geographical Atlas of the USSR (Kolosova 1980).

zone with woodlice records is southern taiga. No woodlice records were in tundra, northern and middle taiga. The species diversity increases southwards, but decreases in the deserts. However, this may be due to the low number of locations extensively studied to reveal local faunas.

Distribution of isopods is known to be limited by natural factors, such as temperature and moisture (Harding and Sutton 1985, Hopkin 1991). In our study, the limiting factor of woodlice distribution towards the north turned out to be the length of the warm period, expressed as number of days when the temperature was above 10°C. The highest species diversity was observed between isoclines of 180 and 210 days with temperature >10°C. Colder conditions slow down their physiological processes (Hopkin 1991) and limit their distribution. For a better understanding of distribution of woodlice, a Species Distribution Modeling (Elith and Leathwick 2009, Franklin 2009) should be applied, which is a next step in the analysis of the database of Russian isopods.

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Appendix 1

List of woodlice species from the territory of the former USSR. Abbreviations: Ab – Abkhazia, Ar – Armenia, Az – Azerbaijan, Bl – Belarus, Ge – Georgia, Kz – Kazakhstan, Kg – Kyrgyzstan, Lt – Lithuania, Md – Moldova, Ru – Russia, Td – Tajikistan, Tu – Turkmenistan, Ua – Ukraine, Uz – Uzbekistan; S, N, W, E – south, north, west, east. References to authorships of the species can be found in Schmalfuss and Wolf-Schwenninger (2002).

1	*Acaeroplastes kosswigi* Verhoeff, 1941	Az: Nabran'
2	*Agabiformius orientalis* (Dollfus, 1905)	Ru: Volgograd region
3	*Armadillidium azerbaidzhanum* Schmalfuss, 1990	Ar: Khastarak; Az: Baku, Adjikend, Drmbon, Lenkoran, Salyany, Sheki; Ge: Kakhetia, Vashlovan; Ru: Rostov, Stavropol regions, N Osetia
4	*Armadillidium granulatum* Brandt, 1833	Ru: Krasnodar region; Ua: S Crimea
5	*Armadillidium nasatum* Budde-Lund, 1885	Ru: Moscow; Ab: Sukhum
6	*Armadillidium opacum* (C.Koch, 1841)	Ua: Kiev
7	*Armadillidium pallasii* Brandt, 1833	Ab: Sukhum; Ua: Crimea, Odessa; Ru: Krasnodar region
8	*Armadillidium pictum* Brandt, 1833	Ua: Crimea
9	*Armadillidium pulchellum* (Zenker, 1798)	Lt: Vilnius
10	*Armadillidium traiani* Demianowicz, 1932	Md
11	*Armadillidium versicolor* Stein, 1859	Ru: Penza, Saratov, Tula regions
12	*Armadillidium vulgare* Latreille, 1804	Ab: Sukhum; Az; Ge: Adygeni, Tbilisi; Ru: Dagestan, Krasnodar, Kaluga, Volgograd, Rostov regions; Ua: Crimea, Kiev, Odessa
13	*Armadillidium zenckeri* Brandt, 1833	Ua: Crimea, Zakaspyisk region
14	*Armadillo alievi* Schmalfuss, 1990	Az: Baku, Kobustan
15	*Armadillo officinalis* Dumeril, 1816	Ru: Krasnodar region; Ua: S Crimea, Odessa
16	*Armadilloniscus ellipticus* (Harger, 1878)	Ru: Krasnodar region; Ab: Gagry
17	*Borutzkyella revasi* (Borutzky, 1973)	Ab: Gudauty region
18	*Buddelundiella cataractae* Verhoeff, 1930	Ge: Tskhaltubo
19	*Caucasocyphonethes cavaticus* Borutzky, 1948	Ru: Krasnodar region
20	*Caucasoligidium cavernicola* Borutzky, 1950	Ab: Gudauty, Sukhum; Ge: Gogolety
21	*Caucasonethes borutzkyi* Verhoeff, 1932	Ge: Tskhaltubo
22	*Chaetophiloscia cellaria* Dollfus, 1884	Ru: Rostov region
23	*Chaetophiloscia elongata* Dollfus, 1884	Ua: Crimea
24	*Chaetophiloscia hastata* Verhoeff, 1929	Ru: Krasnodar region
25	*Colchidoniscus kutaissianus* Borutzky, 1974	Ge: Tskhaltubo
26	*Cylisticoides angulatus* Schmalfuss, 2003	Az: Istisu, Lenkoran, Yardymly; Kz: Astana; Ru: Krasnodar region
27	*Cylisticus albomaculatus* Borutzky, 1957	Ru: Rostov, Voronezh, Volgograd regions
28	*Cylisticus arnoldii* Borutzky, 1961	Ua: Kharkov, Zmiev
29	*Cylisticus birsteini* Borutzky, 1961	Ru: Krasnodar region
30	*Cylisticus caucasius* Verhoeff, 1917	Ab: Gudauty, Gagry, Kelassuri, Sukhum; Ge: Kutaisi, Tkibuli, Tskhaltubo, Shovi; Ru: Krasnodar region

31	*Cylisticus convexus* De Geer, 1778	Ru: Chelyabinsk, Kaluga, Moscow, Rostov, Voronezh regions, Ua: S Crimea, Kiev
32	*Cylisticus cretaceus* Borutzky, 1957	Ru: Rostov region; Ua: Lugansk region
33	*Cylisticus desertorum* Borutzky, 1957	Ru: Rostov, Stavropol regions; Ua
34	*Cylisticus giljarovi* Borutzky, 1977	Ru: Adygea, Stavropol, Krasnodar regions, N Osetia
35	*Cylisticus iners* Budde-Lund, 1880	Ar: Azizbekov, Leninakan, Tshakhkavan; Az: Airidja, Mardakert, Zakatalinsk regions; Ru: Chechnya; Ge: Manglisi, Shuahevi
36	*Cylisticus lencoranensis* Borutzky, 1977	Az: Prishib
37	*Cylisticus mitis* Budde-Lund, 1885	Ge: Kutaisi
38	*Cylisticus orientalis* Borutzky, 1939	Ru: Orenburg region
39	*Cylisticus rotabilis* Budde-Lund, 1885	Ua: S Crimea
40	*Cylisticus sarmaticus* Borutzky, 1977	Ru: Rostov region; Ua: Zaporozhye region
41	*Cylisticus silvestris* Borutzky, 1957	Ru: Moscow, Rostov, Stavropol regions
42	*Cylisticus strouhali* Borutzky, 1977	Ar: W Vanadzor, Spitaki
43	*Desertoniscus birsteini* Borutzky, 1945	Tu
44	*Desertoniscus bulbifrons* Borutzky, 1945	Tu
45	*Desertoniscus elongatus* Borutzky, 1945	Tu
46	*Desertoniscus kirghizicus* Borutzky, 1978	Kg
47	*Desertoniscus reductus* Borutzky, 1978	Td
48	*Desertoniscus subterraneus* Verhoeff, 1930	Kg; Tu: (Kizil-arvat); Td: Samgar massif
49	*Desertoniscus tekinus* Borutzky, 1945	Tu
50	*Desertoniscus zhelochovtzevi* Borutzky, 1945	Uz
51	*Detonella papillicornis* (Richardson, 1904)	Ru: Kamchatka, Sakhalin regions
52	*Halophiloscia couchii* (Kinahan, 1858)	Ru: Krasnodar region; Ua: Crimea
53	*Haplophthalmus danicus* Budde-Lund, 1880	Ru: Krasnodar, Rostov regions
54	*Hemilepistoides messerianus* Borutzky, 1945	Tu
55	*Hemilepistus buddelundi* Borutzky, 1945	Tu
56	*Hemilepistus communis* Borutzky, 1945	Td: Samgar massif
57	*Hemilepistus crenulatus* (Pallas, 1771)	Td: Samgar massif; Tu: Central Karakum, Kyzyl-Arvat; Uz: Zakaspiysk, Fergana regions; Kg
58	*Hemilepistus cristatus* Budde-Lund, 1885	Tu: Kyzyl-Arvat
59	*Hemilepistus elongatus* Budde-Lund, 1885	Ru: Rostov, Stavropol regions; Tu: SW part
60	*Hemilepistus fedtschenkoi* (Uljanin, 1875)	Kz: Semipalatinsk;Tu: Krasnovodsk; Uz: Bukhara, Samarkand, Syrdaryinsk, Zakaspyisk regions
61	*Hemilepistus heptneri* Borutzky, 1945	Tu
62	*Hemilepistus klugii* (Brandt, 1833)	Az: Baku
63	*Hemilepistus magnus* Borutzky, 1945	Uz
64	*Hemilepistus nodosus* Budde-Lund, 1885	Tu; Kz
65	*Hemilepistus pavlovskii* Borutzky, 1954	Kz
66	*Hemilepistus reductus* Borutzky, 1945	Uz: Bukhara, Samarkand, Syrdaryinsk regions
67	*Hemilepistus rhinoceros* Borutzky, 1958	Kz
68	*Hemilepistus ruderalis* (Pallas, 1771)	Ru: Volgograd region; Kz: Djanybek
69	*Hemilepistus russonovae* Borutzky, 1951	Az: Baku
70	*Hemilepistus zachvatkini* Verhoeff, 1930	Td: Samgar massif

71	*Hyloniscus riparius* C. Koch, 1838	Ru: Moscow, Penza, Pskov, Rostov, Tula regions; Ua: Kiev region
72	*Leptotrichus panzerii* (Audonin, 1826)	Ua: Crimea
73	*Leptotrichus tauricus* Budde-Lund, 1885	Ua: Crimea
74	*Ligia cinerascens* Budde-Lund, 1885	Ru: Kurily islands
75	*Ligia italica* Fabricius, 1798	Ua: Crimea
76	*Ligia pallasii* Brandt, 1833	Kadakh?
77	*Ligidium birsteini* Borutzky, 1950	Ab: Gagry
78	*Ligidium cavaticum* Borutzky, 1950	Ru: Krasnodar region
79	*Ligidium fragile* Budde-Lund, 1885	Ab: Sukhum
80	*Ligidium germanicum* Verhoeff, 1901	Md
81	*Ligidium hypnorum* Cuvier, 1792	Ab: Sukhum; Bl: Belovezha National Park; Ru: Tver, Kaluga, Moscow regions; Ua: Crimea, Kiev
82	*Ligidium margaritae* Borutzky, 1955	Kz: Alma-Ata
83	*Ligidium nodulosum* Verhoeff, 1918	Ab: Gagry
84	*Ligidium shadini* Borutzky, 1948	Td
85	*Ligidium tauricum* Verhoeff, 1930	Ua: Crimea
86	*Ligidium zaitzevi* Borutzky, 1950	Ab: Sukhum
87	*Ligidium zernovi* Borutzky, 1948	Kg
88	*Mingrelloniscus inchhuricus* Borutzky, 1974	Ge: Megrelia
89	*Nagurus matekini* Borutzky, 1959	Kg
90	*Oniscus asellus* Linne, 1758	Lt: Vilnius; Ru: Pskov region; Ua: Kiev
91	*Parcylisticus armenicus* Borutzky, 1970	Ar: Daralagez
92	*Parcylisticus dentifrons* Budde-Lund, 1885	Az: Kutkashen; Ge: Manglisi; Ru: Astrakhan, Stavropol regions, Chechnya, Dagestan, Kabardino-Balkaria, N Osetia; Ua: Crimea
93	*Parcylisticus georgianus* Schmalfuss, 2003	Ge: Adigeni, Batumi, Kutaisi, Mestia
94	*Parcylisticus golovatchi* Schmalfuss, 2003	Az: Shikahokh
95	*Parcylisticus mrovdaghicus* (Borutzky, 1970)	Az: Avash, Dashsalty, Kelbadjar, Kirovobad, Lenkoran, Zuvand
96	*Parcylisticus urartuensis* Borutzky, 1970	Ar
97	*Parcylisticus zangezuricus* Borutzky, 1970	Ar
98	*Platyarthrus armenicus* Borutzky, 1976	Ar: Megri
99	*Platyarthrus hoffmannseggii* Brandt, 1833	Ru: Krasnodar region
100	*Platyarthrus luppovae* Borutzky, 1953	Td
101	*Platyarthrus mesasiaticus* Borutzky, 1976	Tu
102	*Platyarthrus ocellatus* Borutzky, 1953	Td
103	*Platyarthrus schoblii* Budde-Lund, 1885	Ua: Crimea
104	*Porcellio bistriatus* Budde-Lund, 1885	Ab: Sukhum; Ru: Krasnodar region
105	*Porcellio crassicornis* C. Koch, 1841	Bl: Minsk
106	*Porcellio dilatatus* Brandt, 1833	Ar: Sevan
107	*Porcellio laevis* Latreille, 1804	Ab: Sukhum; Ru: Altay, Kalmykia, Moscow, Primorie, Rostov regions; Ua: S Crimea, Odessa; Uz: Bukhara region
108	*Porcellio lamellatus* Budde-Lund, 1885	Ua: Crimea
109	*Porcellio obsoletus* Budde-Lund, 1885	Ua: S Crimea

110	*Porcellio scaber* Latreille, 1804	Bl: Belovezha, Berezinsky reserves; Lt: Vilnius; Ru: Belgorod, Kaluga, Moscow, Nizhni Novgorod, Primorie, Rostov regions, Kamchatka, S Kuruly islands, Sakhalin; Ua: Kiev, Kremenetz, Vinnickaya region (Yampol')
111	*Porcellio spinicornis* Say, 1818	Bl: Minsk; Lt: Vilnius; Md; Ru: Kaluga, Leningrad, Moscow Pskov regions; Ua: Kiev
112	*Porcellio uljanini* Budde-Lund, 1885	Ua: Crimea
113	*Porcellio variabilis* Lucas, 1849	Ua: Crimea
114	*Porcellionides approximatus* Budde-Lund, 1885	Md; Ru: Stavropol region; Ua: Crimea
115	*Porcellionides linearis* (Budde-Lund, 1885)	Uz: Nukus
116	*Porcellionides pruinosus* Brandt, 1833	Ab: Sukhum; Ar: Shorzha; Az: Baku, Khachmas, Nabran; Ru: Baikal, Volgograd, Krasnodar region, Moscow, Rostov, Saratov regions; Ua: Crimea
117	*Porcellionides rectifrons* (Budde-Lund, 1885)	Ua: Crimea
118	*Porcellium collicola* (Verhoeff, 1907)	Md
119	*Porcellium conspersum* C. Koch, 1841	Bl: Belovezha Reserve; Ua
120	*Protracheoniscus abricossovi* Borutzky, 1945	Tu
121	*Protracheoniscus alabashensis* Borutzky, 1959	Kg
122	*Protracheoniscus almaatinus* Borutzky, 1975	Kz: Alma-Ata
123	*Protracheoniscus anatolii* Borutzky, 1959	Kg
124	*Protracheoniscus armenicus* Borutzky, 1975	Ge: Megri
125	*Protracheoniscus asiaticus* (Uljanin, 1875)	Ru: Moscow, Nizhni Novgorod, Rostov, Ryazan' regions; Td: Smagar massif
126	*Protracheoniscus atrecicus* Borutzky, 1945	Tu: Bugdaily
127	*Protracheoniscus bugdajliensis* Borutzky, 1975	Tu: Bugdaily
128	*Protracheoniscus cristatus* Borutzky, 1945	Az: Lenkoran, Sara isl.; Tu
129	*Protracheoniscus darevskii* Borutzky, 1975	Ar: Megri
130	*Protracheoniscus delilensis* Borutzky, 1945	Tu
131	*Protracheoniscus desertorum* Verhoeff, 1930	Turkestan?
132	*Protracheoniscus digitifer* Borutzky, 1945	Tu
133	*Protracheoniscus fossuliger* Verhoff, 1901	Ru: Krasnodar, Rostov regions
134	*Protracheoniscus giljarovi* Borutzky, 1957	Ru: Rostov region; Ua: Lugansk region
135	*Protracheoniscus gissarensis* Borutzky, 1975	Td: Dushanbe
136	*Protracheoniscus hirsutulus* Verhoff, 1930	Uz: Tashkent
137	*Protracheoniscus kopetdagicus* Borutzky, 1945	Tu
138	*Protracheoniscus kryszanovskii* Borutzky, 1957	Ru: Volgograd region, Kalmykia
139	*Protracheoniscus latus* (Uljanin, 1875)	Td: Zeravshvan valley
140	*Protracheoniscus litoralis* (Budde-Lund, 1885)	Ua: Crimea
141	*Protracheoniscus major* (Dollfus, 1903)	Ru: Rostov region; Ua: Kiev
142	*Protracheoniscus maracandicus* (Uljanin, 1875)	Td: Smagar massif; Uz: Bukhara, Samarkand, Syrdaryinsk regions
143	*Protracheoniscus marginatus* (Uljanin, 1875)	Ua: Crimea

144	*Protracheoniscus nogaicus* Demianowitz,1931	Md; Ru: Rostov region
145	*Protracheoniscus orientalis* (Uljanin, 1875)	Az: Baku, Mardakert; Bl: Berezinsky reserve; Kz: Mangyshlak; Tu: Bugdaily; Ru: Moscow, Orenburg, Primorie regions; Ua: Kremenetz, Odessa regions, Uz: Nukus; Zakaspyisk region
146	*Protracheoniscus panphilovi* Borutzky, 1959	Kg
147	*Protracheoniscus politus* (C. Koch, 1841)	Ru: Moscow region
148	*Protracheoniscus scythicus* Demianowicz, 1932	Md
149	*Protracheoniscus steinbergi* Borutzky, 1961	TU: SW part
150	*Protracheoniscus taschkentensis* Verhoeff, 1930	Uz: Tashkent; Td
151	*Protracheoniscus tashausicus* Borutzky, 1976	Tu; Ru: Rostov region
152	*Protracheoniscus topczievi* Borutzky, 1975	Ru: Krasnodar, Rostov regions; Ua: Zaporozhye region
153	*Protracheoniscus tuberculatus* (Borutzky, 1945)	Tu
154	*Protracheoniscus turcomanicus* Borutzky, 1945	Tu
155	*Protracheoniscus tzvetkovi* Borutzky, 1975	Kz: Alma-Ata, Uzun-Agach; Ru: Moscow region?
156	*Protracheoniscus uljanini* Borutzky, 1953	Td
157	*Protracheoniscus verhoeffi* Strouhal, 1929	Ge: Tbilisi
158	*Protracheoniscus zenkevitschi* (Borutzky, 1945)	Tu
159	*Psachonethes czerkessicus* Borutzky, 1969	Ru: Krasnodar region
160	*Pseudobuddelundiella hostensis* Borutzky, 1967	Ru: Krasnodar region
161	*Pseudobuddelundiella ljovuschkini* Borutzky, 1967	Ru: Krasnodar region
162	*Schizidium davidi* (Dollfus, 1887)	Az: Divichi
163	*Schizidium golovatchi* Schmalfuss, 1988	Ar: Shikalyukh; Az: Baku; Ge: Batumi
164	*Schizidium reinoehli* Schmalfuss, 1988	Ru: Rostov region
165	*Tadzhikoniscus coecus* Borutzky, 1976	Td
166	*Tauroligidium stygium* Borutzky, 1950	Ua: Crimea
167	*Tauronethes lebedinskyi* Borutzky, 1949	Ua: Crimea
168	*Titanethes albus* (C. Koch, 1841)	Ua: Crimea
169	*Trachelipus azerbaidzhanus* Schmalfuss, 1986	Az: E part
170	*Trachelipus caucasius* (Verhoeff, 1918)	Ab: Gagry; Ru: Krasnodar region
171	*Trachelipus difficilis* Radu, 1950	Bl: Belovezha, Berezinsky reserves; Ua: S Crimea
172	*Trachelipus ensiculorum* Verhoeff, 1949	Ar: Yerevan
173	*Trachelipus gagriensis* (Verhoeff, 1918)	Ab: Gagry
174	*Trachelipus kervillei* (Arcangeli, 1938)	Ru: Rostov region
175	*Trachelipus lignaui* (Verhoeff, 1918)	Ab: Gagry; Ru: Rostov region
176	*Trachelipus longipennis* Budde-Lund, 1885	Ab; Ua: S Crimea
177	*Trachelipus lutschnikii* (Verhoeff, 1933)	Ru: Krasnodar region

178	*Trachelipus rathkii* Brandt, 1833	Ab: Sukhum; Az: Airidja; Ar: Chaldyr; Ge: Kutaisi; Bl: Belovezha pusha; Lt: Vilnius; Md; Ru: Belgorod, Kursk, Tver, Maryi-El, Kaluga, Mordovia, Moscow, Penza, Rostov, Leningrad, Saratov, Tula regions; Ua: Crimea, Kiev
179	*Trachelipus razzautii* (Arcangeli, 1913)	Ru: Krasnodar region
180	*Trachelipus sarculatus* (Budde-Lund, 1896)	Ua: Crimea
181	*Trachelipus trachealis* Budde-Lund, 1885	Md
182	*Trichoniscus aphonicus* Borutzky, 1977	Ab
183	*Trichoniscus gudauticus* Borutzky, 1977	Ab
184	*Trichoniscus pusillus* Brandt, 1833	Ua: Crimea, Kiev
185	*Trichoniscus pygmaeus* Sars, 1898	Ru: Krasnodar region
186	*Turanoniscus anacanthotermitis* Borutzky, 1969	Uz: Tashkent
187	*Tylos granuliferus* Budde-Lund, 1885	Ru: Primorie region, S Kuril Islands
188	*Tylos ponticus* Grebnicki, 1874	Ua: Crimea, Odessa
189	*Typhloligidium coecum* (Carl, 1904)	Ua: Crimea
190	*Typhloligidium karabijajlae* Borutzky, 1962	Ua: Crimea

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Discovery of *Hemilepistus elongatus* Budde-Lund, 1885 (Isopoda, Oniscidea) in Iran: redescription and intraspecific character variability

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Abstract

In the present study, *Hemilepistus elongatus* Budde-Lund, 1885 is reported from Iran for the first time, re-described and its diagnostic characters are figured. This species reveals a high variability in morphological characters. The division of the species at the subspecific level can not be supported anymore. This species differs from other species of the genus by the unique shape of male pleopod-endopodite I.

Keywords

Oniscidea, *Hemilepistus elongatus*, redescription, character variability

Introduction

Budde-Lund (1879) created *Hemilepistus* as a subgenus of the genus *Porcellio* Latreille, 1804. Verhoeff (1930) raised it to the generic level and divided it into two subgenera, *Hemilepistus* and *Desertellio*, based on absence or presence of a frontal line between the profrons and the vertex. Originating in Central Asia (Schmalfuss 1998), this genus has expanded its geographical distribution to North Africa (Lincoln 1970). Recently, Kashani et al. (2010) reported five species of the subgenus *Hemilepistus* from Iran and this is the first record of the subgenus *Desertellio* from this region. According to the species list of Schmalfuss (2003), the subgenus *Desertellio* includes 10 valid species, namely *Hemilepistus buddelundi* Borutzky, 1945, *H. communis* Borutzky, 1945, *H.*

elongatus Budde-Lund, 1885, *H. fedtschenkoi* (Uljanin, 1875), *H. heptneri* Borutzky, 1945, *H. nodosus* Budde-Lund, 1885, *H. pavlovskii* Borutzky, 1954, *H. ruderalis* (Pallas, 1771), *H. russonovae* Borutzky, 1951 and *H. zachvatkini* Verhoeff, 1930.

Hemilepistus elongatus was described by Budde-Lund (1885) on the basis of one female specimen from Taschburun in "Transcaucasus". Borutzky (1945) reported this species from Caucasus and later he (Borutzky1955) described the new subspecies *H. elongatus transcaspius* from Turkmenistan. In addition to the above mentioned localities, Röder et al. (1993, 1996) and Röder and Linsenmayr (1999) reported this species from Ararat, easternmost Turkey. No record of this species has been reported from Iran. The present study, however, showed that this species has a broad geographical distribution in Iran.

Intraspecific variability of morphological characters has been reported in many terrestrial isopods (e.g. in *Oniscus asellus* (Bilton 1994), *Ligidium* spp. (Klossa-Kilia et al. 2006), *Porcellio lamellatus* (Montesanto et al. 2007), *Orthometopon* spp. (Poulakakis and Sfenthourakis 2008), *Armadillo tuberculatus* (Kamilari and Sfenthourakis 2009)). Examination of numerous specimens from different parts of Iran (Fig. 1) and some other specimens from Caucasus revealed that intraspecific variability is present also in some diagnostic characters of *Hemilepistus elongatus*.

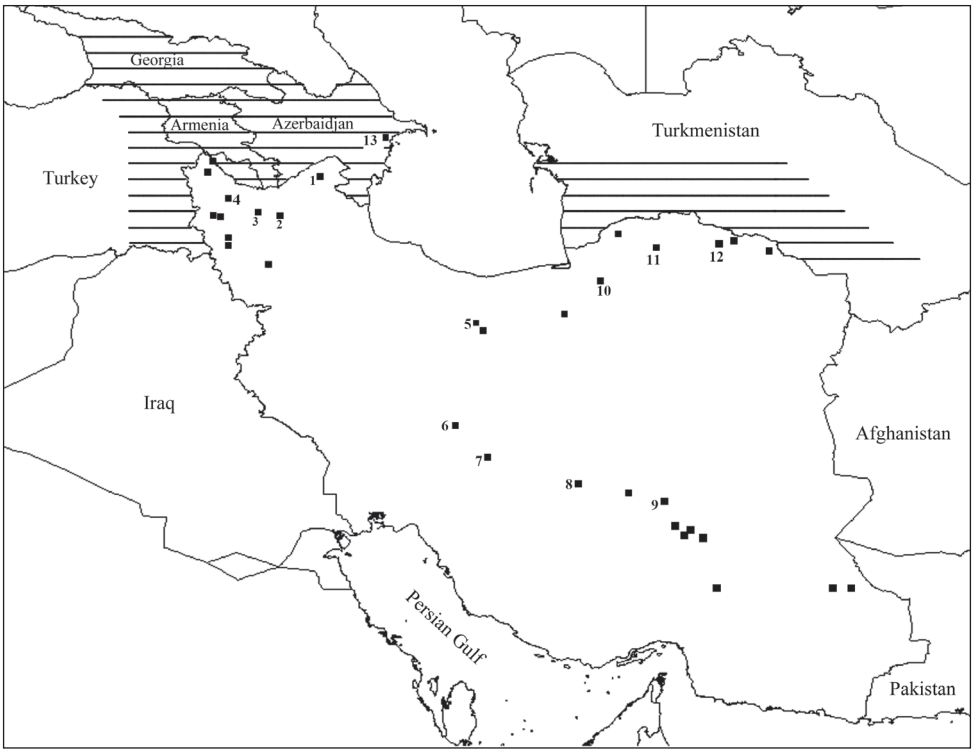

Figure 1. Sampling localities of *Hemilepistus elongatus* from Iran along with its distribution area in other countries (striped line). Numbers refer to the localities in the subsequent figures.

Due to the lack of a comprehensive description along with a critical consideration of subspecific division for the species, the main purposes of the present paper are the redescription of *H. elongatus*, the demonstration of its character variability and the elucidation of its taxonomic status at subspecific level. Moreover, new records of this species from Iran are presented.

Material and methods

The material of the present study from Iran was collected by the first author unless otherwise mentioned. The specimens were collected by hand and preserved in 96% ethanol. The isopods were dissected and body parts were slide-mounted using Euparal (Carl Roth, Karlsruhe). Digital color images were taken using a Qimaging MicroPublisher 5.0 RTV digital camera and Syncroscopy Auto-Montage (v 5.03.0061) software. Drawings were made using a camera lucida fitted on an Olympus SZX12 dissecting stereomicroscope and on an Olympus BX51 compound microscope. The material used for SEM-preparations was air-dried overnight. The mounted material was coated with gold in a sputter coater to 40 nm thickness and examined with a Hitachi S-2460N SEM.

For comparison, type or additional material was obtained from the Natural History Museum, London (BMNH) and Staatliches Museum für Naturkunde, Stuttgart (SMNS). The examined material from Iran is deposited in the Zoological Museum, University of Tehran (ZUTC). Some specimens are kept in the personal collection of the first author.

Results and discussion

In addition to the presence of *H. elongatus* in Caucasus, Turkey and Turkmenistan, the present study confirms the occurrence of this species from Iran where it has a broad geographical distribution (Fig. 1) in diverse habitats. This species, like many other terrestrial isopods, shows a high variability in many morphological characters including coloration, size and shape of frons, ratio of flagellar articles, shape of pleotelson, and even in the male secondary sexual structures such as pereiopod VII ischium and pleopod I (see description). This character variability led Borutzky (1955) to propose a new subspecies, *H. elongatus transcaspius,* for specimens from Turkmenistan. According to the Russian author, this subspecies differed from the typical *H. elongatus* in the shape of the pleopod-endopodite and exopodite I. Evaluation of numerous specimens from different localities in Iran and Caucasus revealed that various states of these characters are sometimes found within the same population, often with intermediate forms. Though there was no possibility to examine specimens from Turkmenistan, the differences which led Borutzky (1955) to describe a new subspecies, fall within the morphological variability of *H. elongatus*.

Family Agnaridae Schmidt, 2003

***Hemilepistus elongatus* Budde-Lund, 1885**
http://species-id.net/wiki/Hemilepistus_elongatus

Hemilepistus elongatus Budde-Lund, 1885: 160.– Walter 1889: 1110.– Röder et al. 1996: 818.– Ziegler and Miller 1997:181.– Röder and Linsenmayr 1998: 57.– 1999: 349.– Jeppesen 2000:238.
Hemilepistus (Desertellio) elongatus Borutzky 1945:198.
Hemilepistus (Desertellio) elongatus transcaspius Borutzky 1955: 217.– 1961: 26
Desertellio elongatus Röder et al., 1993: 339.

Material examined. Iran: Marand to Ghare-Ziaoddin, 38˚35.5'N, 45˚15.7'E, 8 November 2004, leg. A. Kazemi, one male and one female (ZUTC Iso.1080); Bilesavar to Parsabad, 39°33.8'N, 47°56.7'E, 16 June 2008, one male and one female (ZUTC Iso.1081); Tabriz to Khaje, 38°08.7'N, 46°35.7'E, 17 June 2008, one male and one female (ZUTC Iso.1082); Tabriz to Marand, 38°14.9'N, 47°06.6'E, 18 June 2008, one female (ZUTC Iso.1083); Poldasht to Makoo, 39°17.0'N, 44°42.7'E, 18 June 2008, one female (ZUTC Iso.1084); Urmia, Golmankhaneh port, 37°35.7'N, 45°15.3'E, 2 October 2008, one male and two females (ZUTC Iso.1085); Shahin-Dezh to Miandoab, 36°52.5'N, 46°17.3'E, 3 October 2008, two males and two females (ZUTC Iso.1086); Tabriz, Agh-Gonbad port, 17 June 2008, one female (ZUTC Iso.1087); Shirvan, 37°25.1'N, 57°52.7'E, 6 May 2008, one male (ZUTC Iso.1088); Abadeh to Semirom, 31°05.8'N, 51°56.3'E, 9 April 2008, one male and one female (ZUTC Iso.1089); Varamin, Pishva, 35°12.2'N, 51°48.2'E, 23 June 2008, one female (ZUTC Iso.1090); Zahedan to Khash, 28°32.8'N, 60°49.4'E, 28 February 2009, one female (ZUTC Iso.1091).

Additional material. Turkey: Holotype, female, Caucasus, Taschburun, in A. Brandt collection, leg.?, det. Budde-Lund (BMNH 1921.10.18–4103); **Turkmenistan:** Tschikischljar, 27 April 1986, leg.?, one female (BMNH 1921.10.18–4102); **Azerbaidjan:** S. Baku, 20 km N Salyani, 30 May 1996, leg. W. Schawaller, det. H. Schmalfuss, one male and one female (SMNS 11530); **Georgia:** Caucasus, Vashlovan Reserve, 7–9 May 1983, leg. Golovatch, det. H. Schmalfuss, one male and one female (SMNS 13082); **Iran:** 150 km N Isfahan, 4 June 1975, leg. Bauer, det. H. Schmalfuss, one female (SMNS 11020).

Diagnosis. Cephalothorax with rounded lateral lobes and short to developed median lobe, frons with or without incision in the middle; dorsal part with several rounded tubercles of the same or different size. Pereion-tergites I to III with tubercles decreasing in number posteriorly. Male pereiopod VII ischium with straight to sinuate ventral margin. Male pleopod-endopdite I straight; apex with a leaf-like lobe.

Redescription. Maximum length of both male and female: 18 mm. Body elongated. Color brown with epimera, posterior margin of tergites and pleotelson pale (Fig. 2).

Cephalothorax with rounded lateral lobes, median lobe with variable size and shape; several rounded tubercles of the same or different size in dorsal part (Fig. 3A–D); fron-

Figure 2. *Hemilepistus elongatus* from Isfahan, Semirom to Abadeh (7). Female, dorsal view and lateral view of head and first four pereionites. Scale, 2 mm.

tal line sinuous in frontal view, with or without incision in the middle; no suprantennal line (Fig. 3E–F); eyes with 20–25 ommatidia. Antenna long, reaching posterior margin of second pereion-tergite; flagellum slightly shorter than fifth article of peduncle, with two articles, first article of equal length or up to 2.5 times as long as second article (Fig. 4A). Antennule of three articles with a tuft of long aesthetascs at apex (Fig. 4B).

Pereion-tergite I with rounded tubercles, two markedly larger tubercles on the median part; and with rounded hind margin (Fig. 5I). Pereion-tergites II–III with fewer tubercles. Pereion-tergites IV-VII smooth.

Pleon short, smooth, slightly narrower than pereion (Fig. 4C). Pleotelson short, triangular, with slightly concave sides and acute, rounded or truncate apex slightly surpassing uropod-protopodites (Fig. 5J). Uropod-exopodites conical, about twice as long as protopodites

Pleopod-exopodites I–V with monospiracular covered lungs.

Male: Pereiopod I merus and carpus with or without brushes of setae (Fig. 4D). Pereiopod II to VII with no brushes of setae on merus and carpus. Pereiopod VII ischium with straight or sinuate ventral margin; merus and carpus equipped with strong setae (Fig. 4E).

Pleopod-endopodite I straight, apex with a leaf-like lobe, equipped with setae, variable in shape (Fig. 5A). Pleopod-exopodite I as in Fig. 5B–F, inner lobe variable in shape. Pleopod-exopodites II-III as in Fig. 5G–H.

Remarks. This species is distinguished from other species of the genus by the unique shape of male pleopod-endopodite I, with apex bearing a leaf-like lobe.

Distribution. Georgia; Azerbaijan; easternmost Turkey; Turkmenistan; Iran.

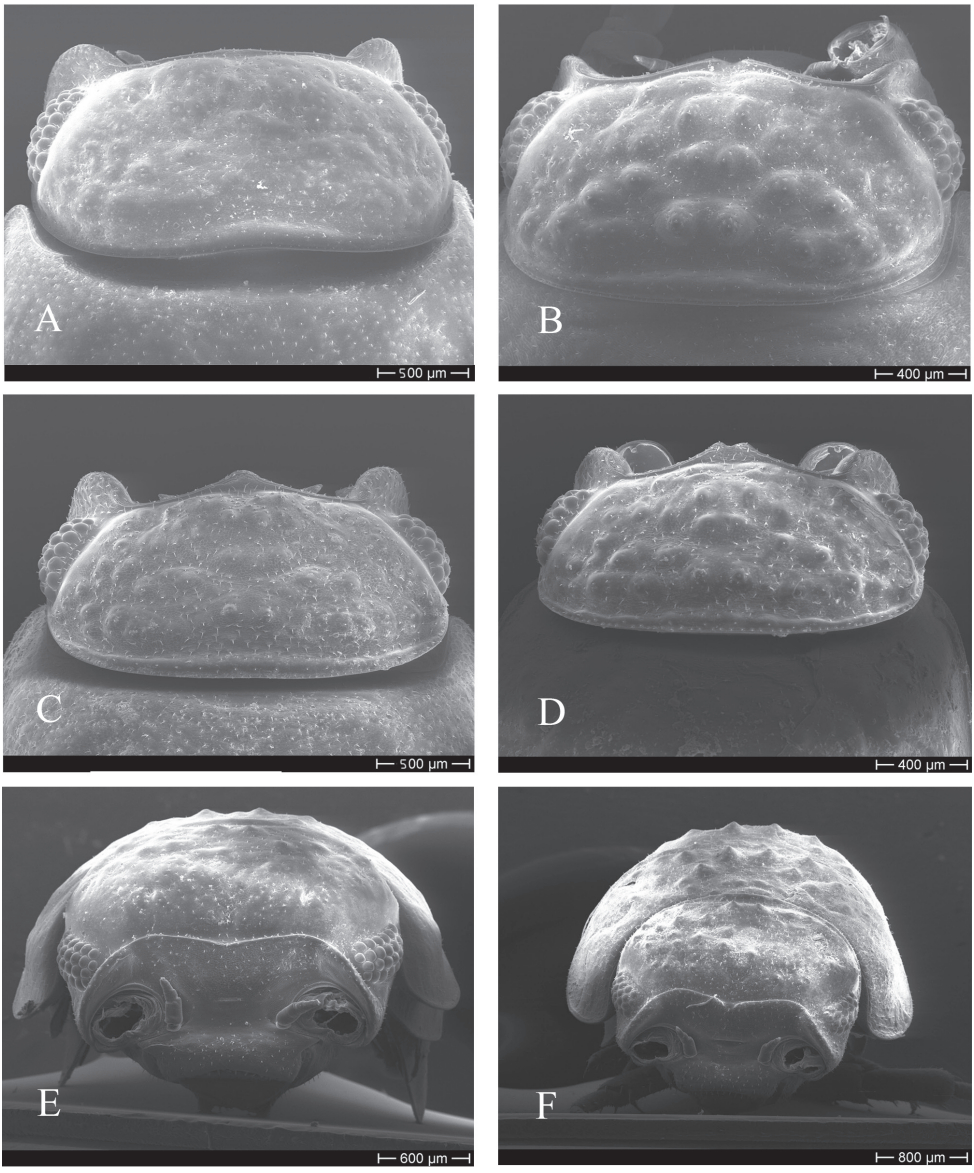

Figure 3. *Hemilepistus elongatus*. Cephalothorax; **A–D** dorsal view; **E–F** frontal view **A** from Kerman, Bardsir (9), 12 mm long **B** from Khorasan, Shirvan (12), 12 mm long **C** from Alborz, Karaj (5), 13 mm long **D** from Tabriz, Soofian (3), 11 mm long **E** from Kerman, Bardsir (9), 14 mm long; **F** from Tabriz, Marand (4), 14 mm long.

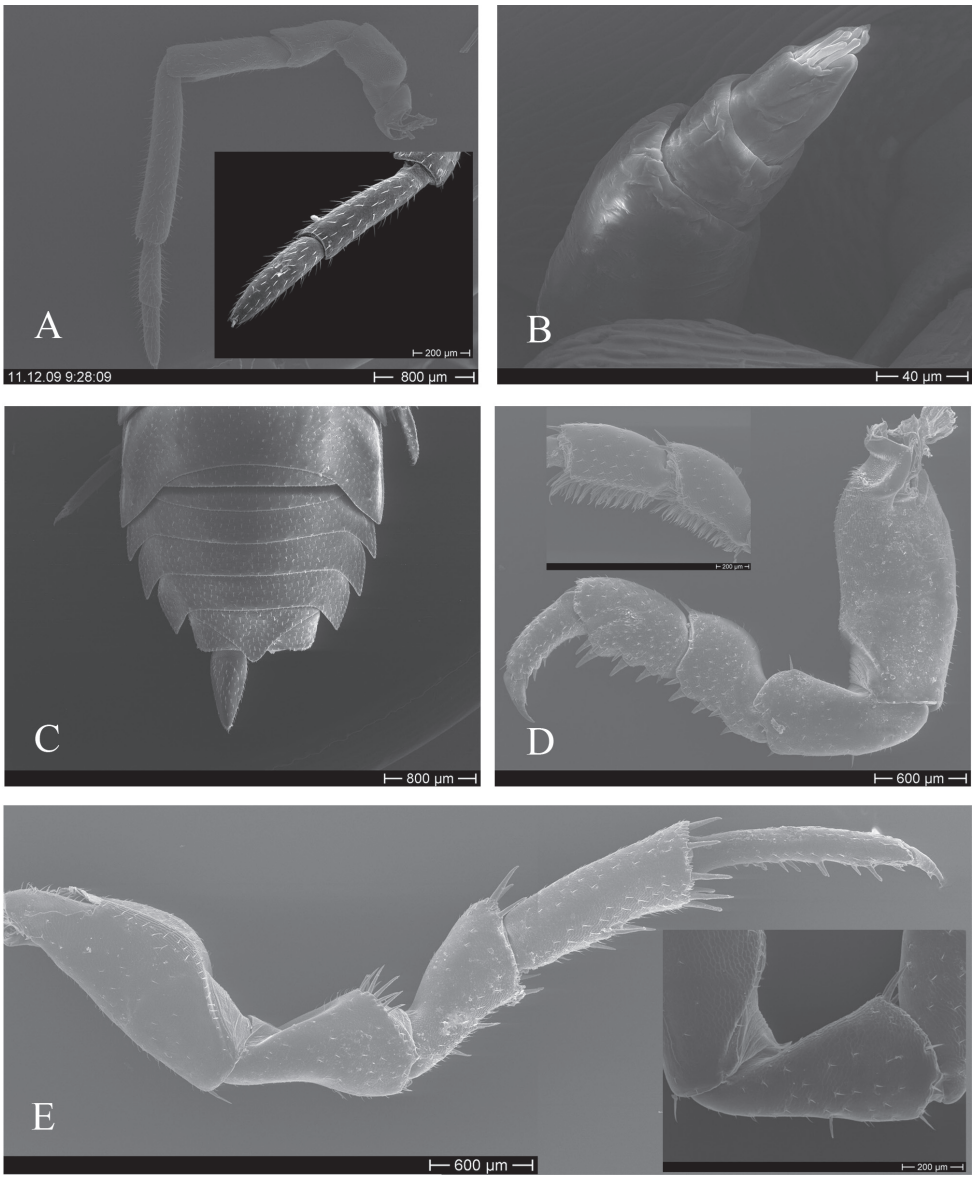

Figure 4. *Hemilepistus elongatus*. **A** antenna **B** antennule **C** pleon **D** male pereiopod I from Khorasan, Shirvan (12) and Ardabil, Parsabad (1) (merus & carpus) **E** male pereiopod VII from Ardabil, Parsabad (1) and Tabriz, Soofian (3) (ischium).

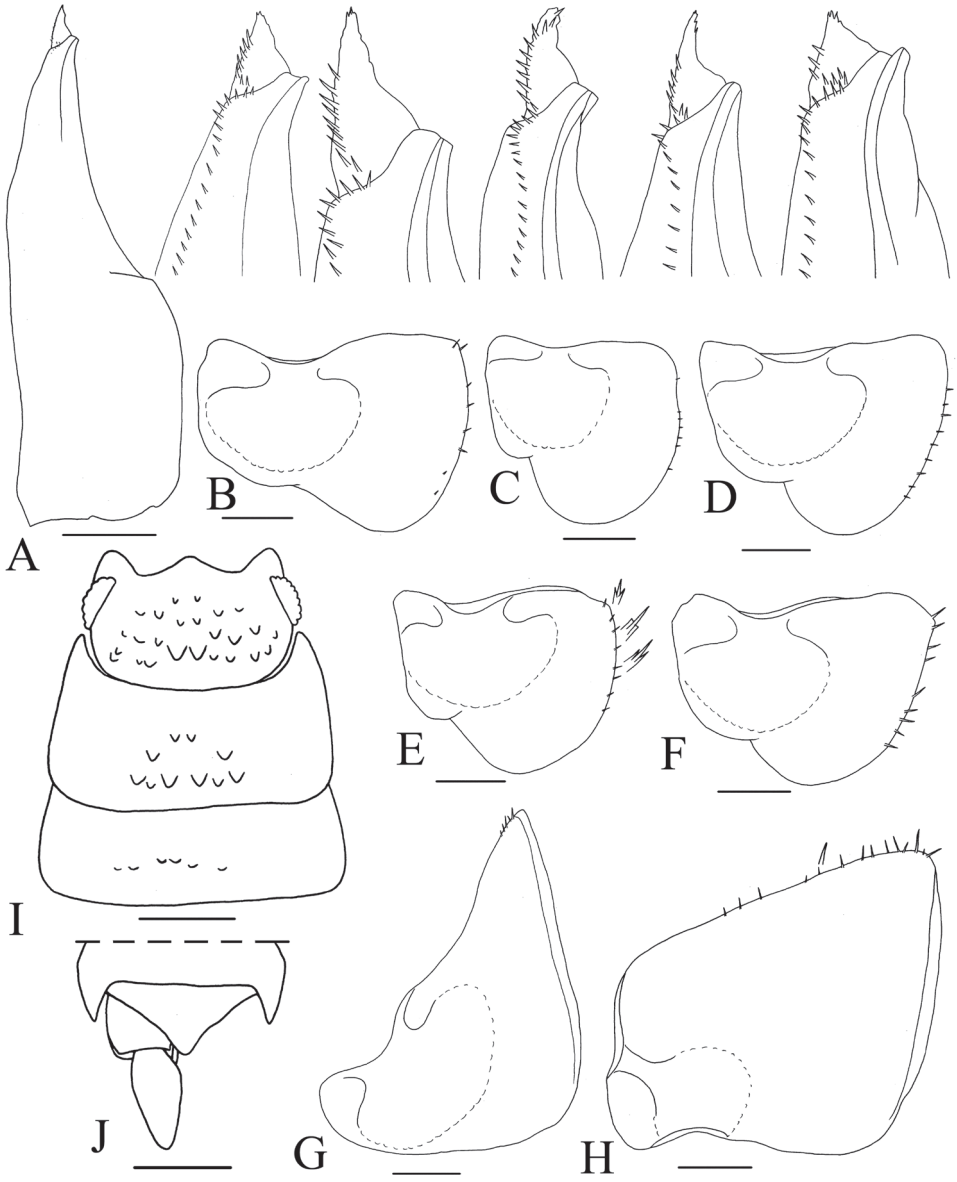

Figure 5. *Hemilepistus elongatus*. **A** male pleopod-endopodite I and five enlarged apex, left to right from Azerbaijan, S Baku (13), Isfahan, Tiran (6), Fars, Saadatshahr (8), Semnan, Kalate-Khij (10) and Ardabil, Parsabad (1) **B–F** male pleopod-exopodite I **B** from Northen Khorasan, Ashkhaneh to Minoo-Dasht (11) **C** from Ardabil, Parsabad (1) **D** from Tabriz, Khajeh (2) **E** from Azerbaijan, S Baku (13) **F** from Isfahan, Semirom to Abadeh (7) **G** male pleopod-exopodite II, from Azerbaijan, S Baku (13) **H** male pleopod-exopodite III, from Azerbaijan, S Baku (13) **I holotype**, cephalotorax and pereion-tergites I-II **J holotype**, pleotelson. Scales, 0.5 mm for **A–H** and 1 mm for **I-J**.

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Molting and cuticle deposition in the subterranean trichoniscid *Titanethes albus* (Crustacea, Isopoda)

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Abstract

Terrestrial isopods are a suitable group for the study of cuticle synthesis and calcium dynamics because they molt frequently and have evolved means to store calcium during molt. Little data is currently available on molting in Synocheta and subterranean isopods. We studied the molting dynamics in the subterranean trichoniscid *Titanethes albus* under laboratory conditions and performed a microscopic investigation of sternal $CaCO_3$ deposits and the tergal epithelium during molt in this species. In accordance with its lower metabolic rate, molting in the laboratory is roughly 2–3 times less frequent in *T. albus* than would be expected for an epigean isopod under similar conditions. Animals assumed characteristic postures following the molt of each body half and did not consume the posterior exuviae after posterior molt. The structure of sternal calcium deposits and the ultrastructural characteristics of the epidermis during cuticle formation in *T. albus* are similar to those described in representatives of Ligiidae. During the deposition of the exocuticle, the apical plasma membrane of epidermal cells forms finger-like extensions and numerous invaginations. In the ecdysial space of individuals in late premolt we observed cellular extensions surrounded by bundles of tubules.

Keywords

Cuticle ultrastructure, troglobite, calcium storage

Introduction

Terrestrial isopods are known to molt frequently throughout their life cycle, making them particularly suitable for the study of cuticle synthesis and mineralization (Price and Holdich 1980). Furthermore, the onset of premolt can be easily determined in many terrestrial isopods due to the appearance of sternal calcium deposits (Zidar et al. 1998).

Isopods molt in two phases, first shedding the posterior and then the anterior half of the body. The boundary between the two halves is between pereionites 4 and 5. This pattern of biphasic molt is convenient and enables the simultaneous observation of the integument just prior to molt in the anterior half and just after molt in the posterior half of the same specimen.

Within Oniscidea, several studies have dealt with the ultrastructural aspects of cuticle deposition in Ligiidae (Glötzner and Ziegler 2000, Štrus and Blejec 2001) and in some members of the most terrestrial group, Crinocheta (Price and Holdich 1980, Compere 1990, Ziegler 1997). The ultrastructure of sternal $CaCO_3$ deposits has been analyzed in representatives of Ligiidae and several species of Crinocheta (Ziegler and Miller 1997). Their composition and formation have been studied in great detail in *Porcellio scaber* (reviewed in Ziegler et al. 2005). Data on cuticle synthesis in Synocheta are lacking, although there is some morphological information on calcium storage in this group (Verhoeff 1926, Ziegler 2003).

Caves are stabile but nutrient poor habitats characterized by constant temperature corresponding to the average year temperature on the surface, permanent darkness and near-saturated relative humidity of air. Troglobitic animals have evolved specific adaptations to this environment, such as reduced pigmentation, thin cuticles, and lowered metabolism (Romero and Green 2005). *Titanethes albus* (C. Koch) is a large (about 1.5 cm in length) troglobitic representative of the family Trichoniscidae. The species inhabits wet limestone caves in the Dinaric Karst (Strouhal 1939) and is not exclusively terrestrial, as it is known to enter the water and can survive submerged for long periods (Sket 1986). The tergal cuticle of *T. albus* is thin compared to non-troglobitic isopods of similar size. It is also less mineralized and differs from the cuticles of non-troglobitic oniscids in its mineral composition, having a lower content of magnesium and calcite (Hild et al. 2009).

In our study, we observed the temporal dynamics of molt in a laboratory culture of *T. albus*. We provide an ultrastructural description of tergal cuticle deposition in this species and describe the characteristics of its sternal $CaCO_3$ deposits.

Methods

Laboratory culture and molt cycle observations

Specimens of *T. albus* from caves in central Slovenia were kept in the speleobiological laboratory at the Department of Biology, University of Ljubljana. The laboratory culture was maintained in a dark climate chamber at 11 ± 1 °C, the approximate average temperature of caves in central Slovenia. Animals were kept in glass containers with flowstone rocks, substrate from the sampling sites and spring water. Decaying wood and carrots were provided as food.

Individuals in culture were inspected for sternal deposits every month. Animals with sternal deposits were isolated into Petri dishes containing wet filter paper and observed daily. Every week, the sternal deposits were observed under a stereomicroscope

and their shape was drawn. After the first molt, specimens that were not fixed for microscopic examination were kept individually separated and were inspected weekly for the presence of sternal deposits in order to determine the onset of the following premolt.

Light microscopy and transmission electron microscopy

For ultrastructural observations, animals in premolt (determined by the presence of sternal deposits), intramolt (between the posterior and anterior molt), postmolt (1–2 days after the anterior molt), and intermolt were fixed. Individuals without sternal deposits that did not molt in the previous three weeks were considered to be in the intermolt stage.

Animals were dissected and isolated anterior tergites were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH = 7.3) at 4 °C for at least a week. Specimens were postfixed with 1% OsO_4 for 1 hour, dehydrated in a graded ethanol series and embedded in Spurr's resin. Semithin (0.5 μm) sections were transferred to polylysine coated slides, stained with a mixture of Azur II and Methylene blue (Richardson et al. 1960) and imaged with an AxioImager Z.1 microscope (Zeiss) equipped with an HRc Axiocam camera. Thin (70 nm) sections were collected on copper grids, contrasted with uranyl acetate and lead citrate and examined with a CM 100 transmission electron microscope (FEI). Electron micrographs were recorded with a 792 BioScan camera (Gatan).

Scanning electron microscopy of sternal deposits

In preparation for scanning electron microscopy of sternal deposits, sternites of pereionites 1–4 with fully developed $CaCO_3$ deposits from premolt and intramolt specimens were removed, immersed in methanol and then air dried. When dry, the sternites were attached to aluminum holders and cleaved on an ultramicrotome with a glass knife. Samples were then sputter coated with platinum and imaged with a JSM-7500F field emission scanning electron microscope (JEOL).

Results

Duration of molt cycle

In the individuals studied, the median premolt duration (measured from the appearance of sternal deposits to the onset of molt) was 7 weeks (N=10). The shortest and longest premolt lasted 4 and 9 weeks, respectively. The median length of the period between the completion of molt and the second appearance of sternal deposits in non-ovigerous individuals was 11 weeks (N=9), with extreme values of 9 and 19 weeks. The only observed intermolt period of an ovigerous female lasted 34 weeks, with the release

of brood after 30 weeks. The anterior ecdysis followed 3 to 5 days after the posterior ecdysis. A diagrammatic representation of a typical molt cycle is provided in Fig. 1A. Some processes indicated on the diagram will be explained later in text.

After molting the posterior exuvium, the animals hold the newly molted body-half upwards so that it does not touch the substrate (Fig. 1B). The posterior three pairs of pereiopods lie closely appressed against the ventral body surface and the entire body is supported by the anterior four pairs of pereiopods. Animals maintain this posture for several hours, but they begin using the posterior pereiopods before the onset of anterior molt. After the anterior molt, the anterior body-half is held upwards in a similar manner, with the body now supported by the posterior three pairs of pereiopods (not shown).

In the laboratory, animals never consume their posterior exuviae during intramolt and no part of the shed exoskeleton is consumed directly upon the completion of molt. Exuviae (mostly of the posterior body-half), demonstrably belonging to *T. albus* due to the presence of gland-piliferous organs on the fourth pleonite, were also found on rocks in sampling localities (Fig. 1C), indicating that animals relinquish their old exoskeletons in nature as well.

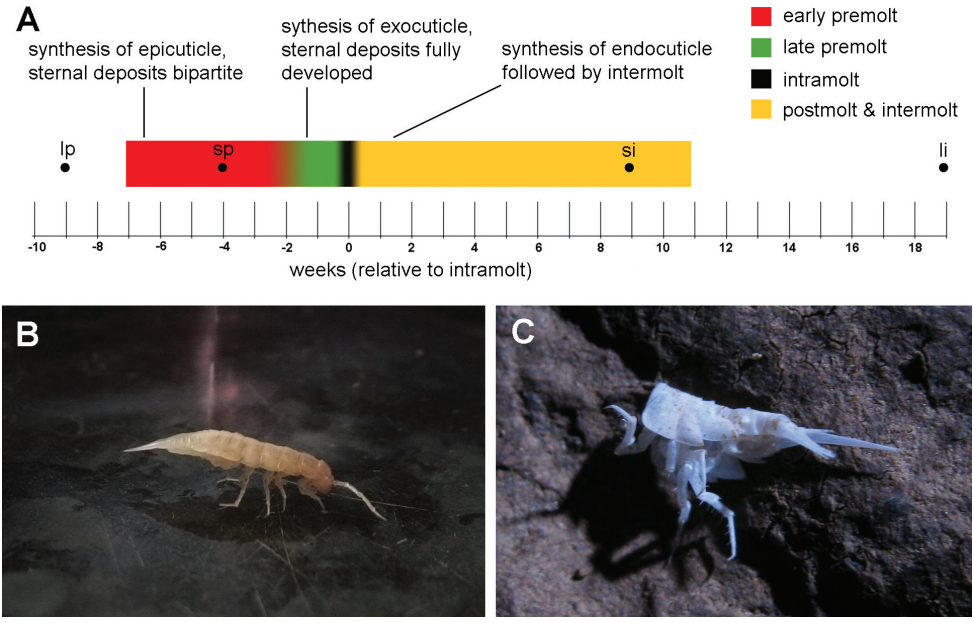

Figure 1. Molting in *Titanethes albus*. **A** a diagram of a typical molt cycle. The colored line shows the median observed durations of premolt and postmolt with intermolt. Different colors represent individual stages in the molt cycle. Key processes in each stage (early premolt, late premolt, and postmolt with intermolt) are indicated. Black dots indicate the onset of the longest and shortest observed premolt stages and the end of the longest and shortest observed intermolt stages **B** *T. albus* immediately after posterior molt. The posterior half of the body is held upwards while the body is supported solely by the anterior four pairs of pereiopods **C** the posterior exuviae of *T. albus* on a rock in Viršnica Cave. **li** end of longest observed intermolt stage **lp** onset of longest observed premolt stage **si** end of shortest observed intermolt stage **sp** onset of shortest observed premolt stage.

Sternal $CaCO_3$ deposits

Like other oniscids, *T. albus* develops sternal $CaCO_3$ deposits in the ecdysial space of the anterior four sternites in premolt. Initially, the deposits are bipartite, with an anterior and a posterior part on each of the first four sternites of the pereion. The shape of sternal deposits in early premolt varies greatly between individuals. The anterior part of individual deposits is always larger and symmetrical (Fig. 2A), whereas the posterior part is smaller and irregular in shape. Sternal deposits in early premolt often display small round fenestrations (Fig. 2A). The location of these fenestrations on the deposits is highly variable. After their initial appearance, the shape of sternal deposits remains unaltered throughout most of the premolt stage. Towards the end of this stage, their shape changes rapidly, the two parts of each deposit fuse and the deposits assume a characteristic, uniform shape (Fig. 2B) in most individuals. Animals molt within a week after this change in the shape of the sternal deposits.

As revealed by scanning electron microscopy, fully formed sternal deposits of *T. albus* are composed of spherules, most of which measure 0.3 μm in diameter and vary in size between 0.1 μm and 1 μm (Fig. 2C). Deposits become progressively more compact from the sternal epidermis towards the old cuticle and spherules in the distal parts of deposits appear fused (Fig. 2D).

Cuticle deposition

Early premolt

The onset of apolysis is observable in the anterior tergites of animals in which the sternal deposits have just appeared. The epidermis and the old cuticle are in close proximity, but protrusions of the apical plasma membrane of epidermal cells with dense tips are already evident within the narrow ecdysial space (Fig. 3A). Later, the ecdysial space is wide and a fibrous and finely granular sheet is present in its distal part (Fig. 3B). The new epicuticle is initially synthesized as a thin electron dense layer over the short protrusions of the apical plasma membrane (Fig. 3C). Gaps in the epicuticle are visible in early stages of its synthesis, indicating that it is discontinuous in the initial stages of its deposition. Oblique sections through the apical epidermal surface suggest that the gaps are perforations of the epicuticle (Fig. 3D). Epicuticular protrusions (scales or hairs) begin to form around cell projections (Fig. 3E), but are initially flat and thinner than the corresponding epicuticular structures in intermolt. Epidermal cells possess a well developed rough endoplasmic reticulum (RER) (Fig. 3E).

Late premolt

In animals with fully formed sternal deposits, lamellae of the exocuticle are being deposited. During the synthesis of the distal dense layer (cf. Hild et al. 2009), the apical plasma membrane of epidermal cells forms finger-like extensions in addition to short

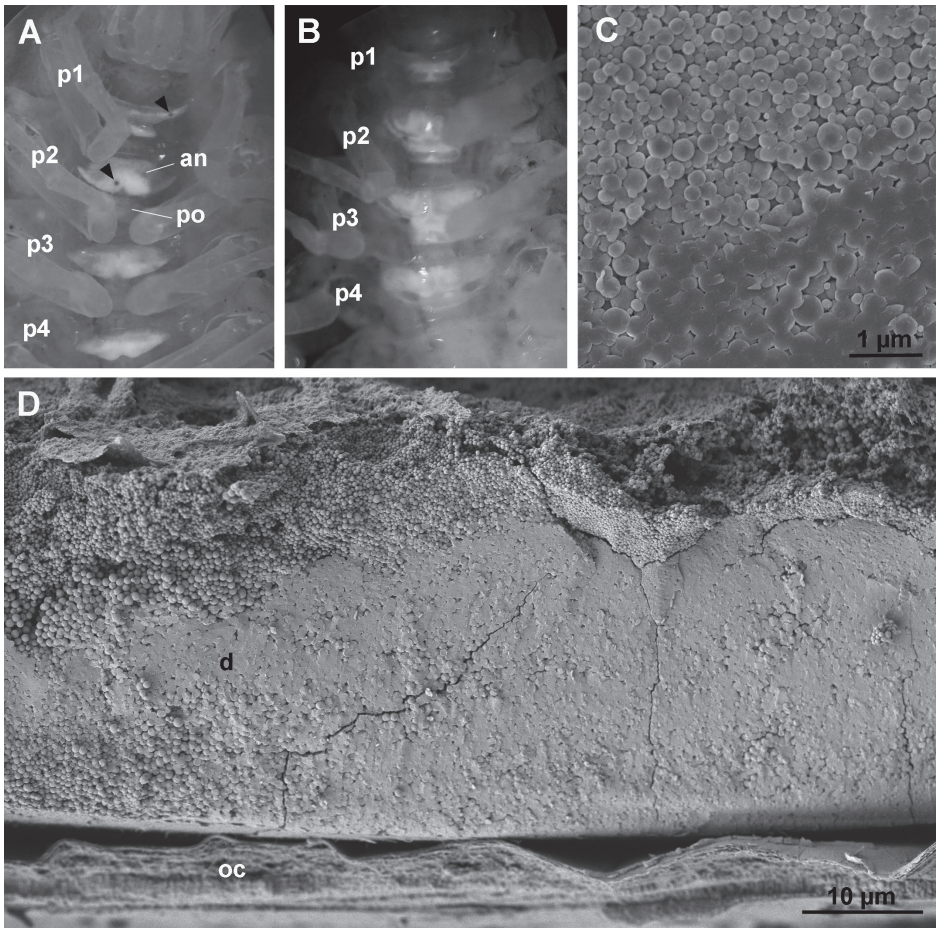

Figure 2. Sternal $CaCO_3$ deposits in *Titanethes albus*. **A** individual in early premolt with sternal deposits on anterior four pereionites. Deposits are bipartite with a larger anterior part (**an**) and a smaller posterior part (**po**). Round fenestrations (arrowheads) perforate the deposits. **B** individual in late premolt with fully developed sternal deposits. The anterior and posterior part on each segment are fused and the deposits have a uniform shape. **C** scanning electron micrograph of spherules forming the sternal deposits in late premolt. **D** scanning electron micrograph of a cleaved sternal deposit (**d**) in late premolt. Spherules are proximally more loosely arranged. **p1** pereionite 1, **p2** pereionite 2, **p3** pereionite 3, **p4** pereionite 4, **oc** old cuticle.

protrusions (Fig. 4A). The Golgi apparatus is well developed in addition to the RER (Fig. 4A). At this stage, the epicuticle and the structures it forms are already fully developed (Fig. 4A, B).

When the exocuticle consists of 3–5 lamellae, small electron dense vesicles appear in the apical cytoplasm of epidermal cells with well developed RER and numerous mitochondria (Fig. 4B). At this stage, the apical plasma membrane of epidermal cells forms numerous invaginations in addition to protrusions (Fig. 4C). Long cytoplasmic

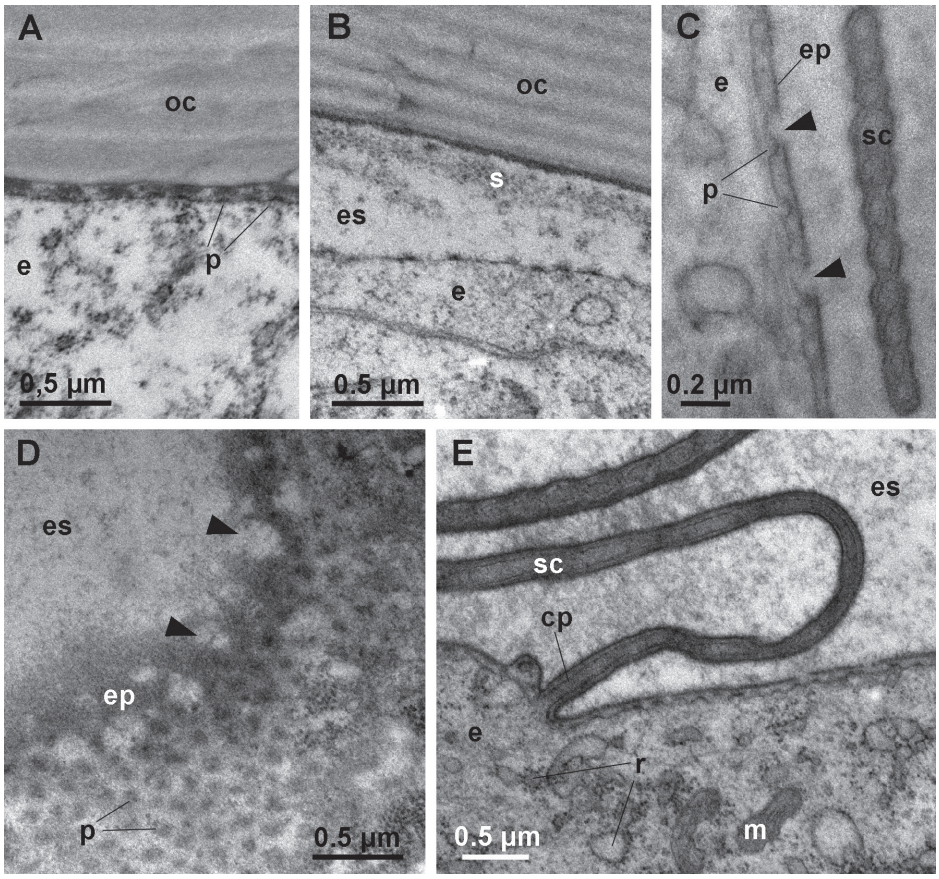

Figure 3. Ultrastructure of anterior tergites in early premolt. **A** apolysis. The apical surface of the epidermal cell (**e**) is detached from the old cuticle (**oc**), but the ecdysial space is narrow. The apical plasma membrane of epidermal cells forms short protrusions (**p**) with electron dense tips. **B** the ecdysial space in early premolt. A sheet of fibrous and granular material (**s**) is located in the distal part of the ecdysial space (**es**). **C** section through the apical surface of an epidermal cell (**e**) in early premolt. Gaps (arrowheads) are present in the newly formed epicuticle (**ep**) that is formed over short protrusions (**p**) of the apical plasma membrane. A developing epicuticular scale (**sc**) is visible. **D** oblique section through the apical surface of an epidermal cell in early premolt. Gaps (arrowheads) in the newly deposited epicuticle (**ep**) appear to be perforations. **E** the epidermis in early premolt. Epidermal cells (**e**) contain numerous mitochondria (**m**) and a well developed RER (**r**). Scales (**sc**) are forming around elongated projections of the apical plasma membrane (**cp**).

extensions reaching into pore canals and extending to the distal dense layer of the new exocuticle become evident (Fig. 4C).

In intramolt, as the anterior tergites are nearing molt, the new exocuticle approaches its final thickness (Fig. 4D). Epidermal cells maintain the characteristics of the late premolt stage with small electron dense vesicles in the apical cytoplasm and a well developed RER (Fig. 4D). Cytoplasmic extensions in pore canals are prominent (Fig. 4D, E) and the apical plasma membrane still forms numerous short protrusions with dense tips (Fig. 4E).

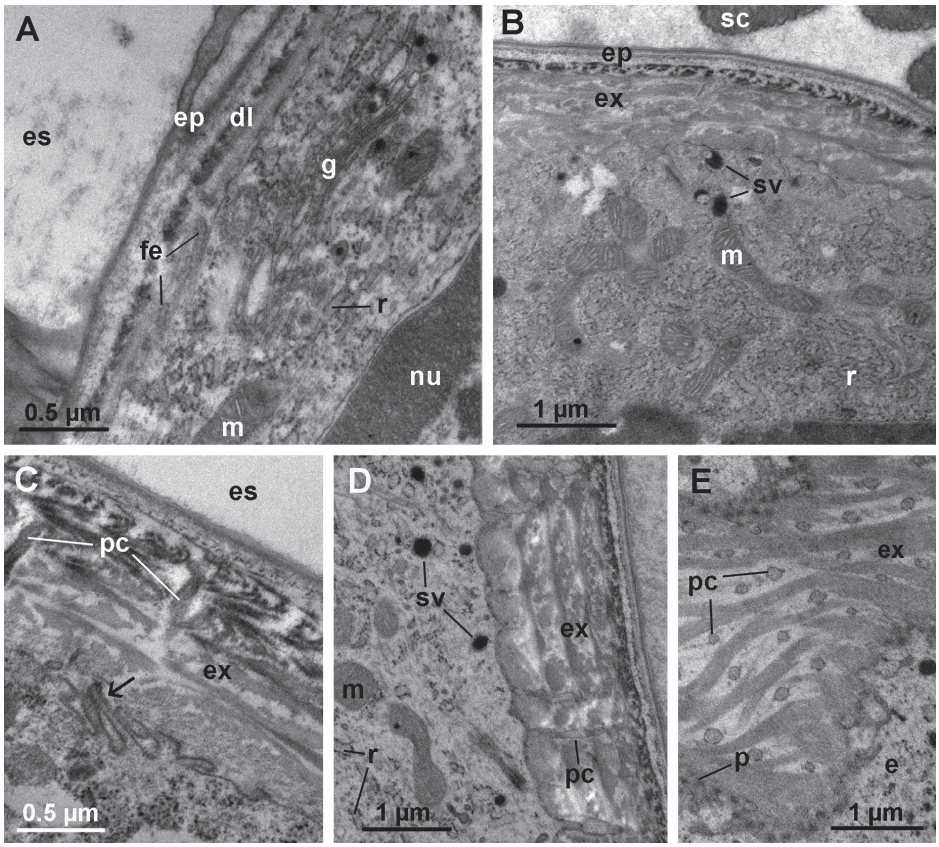

Figure 4. Ultrastructure of anterior tergites in late premolt and intramolt. **A** early stage of exocuticle formation. The distal dense layer (**dl**) is deposited. The apical plasma membrane of epidermal cells forms finger-like extensions (**fe**). The Golgi apparatus (**g**) is well developed. **B** anterior tergite in late premolt. Several lamellae of the new exocuticle (**ex**) are deposited. Epidermal cells contain a well developed RER (**r**), numerous mitochondria (**m**) and small electron dense vesicles (**sv**) in their apical cytoplasm. The epicuticle (**ep**) with scales (**sc**) is fully formed. **C** the apical plasma membrane of an epidermal cell in late premolt forms numerous invaginations (arrow). Cytoplasmic extensions reach into pore canals (**pc**). **D** anterior tergite in intramolt. The new exocuticle is almost fully deposited. The apical cytoplasm of epidermal cells contains electron dense vesicles (**sv**), numerous mitochondria (**m**) and a well developed RER (**r**). Long cytoplasmic extensions reach into pore canals (**pc**). **H** oblique section through the apical surface of an epidermal cell in intramolt. Pore canals (**pc**) in the new cuticle contain cytoplasmic extensions of epidermal cells. Numerous short protrusions (**p**) of the apical plasma membrane with dense tips are visible. **es** ecdysial space. **nu** nucleus.

During late premolt and intramolt stages, tubular bundles are present in the ecdysial space (Fig. 5A, B). The tubules, each measuring about 20 nm in diameter, protrude from the epicuticle in a regular arrangement and are covered by a diffuse electron dense material (Fig. 5B, C). Towards the old cuticle the regular arrangement of tubules is lost and the tubules disperse (Fig. 5B). At the center of each bundle of tubules there is a cellular extension containing parallel microtubules. The extension is enclosed in an elec-

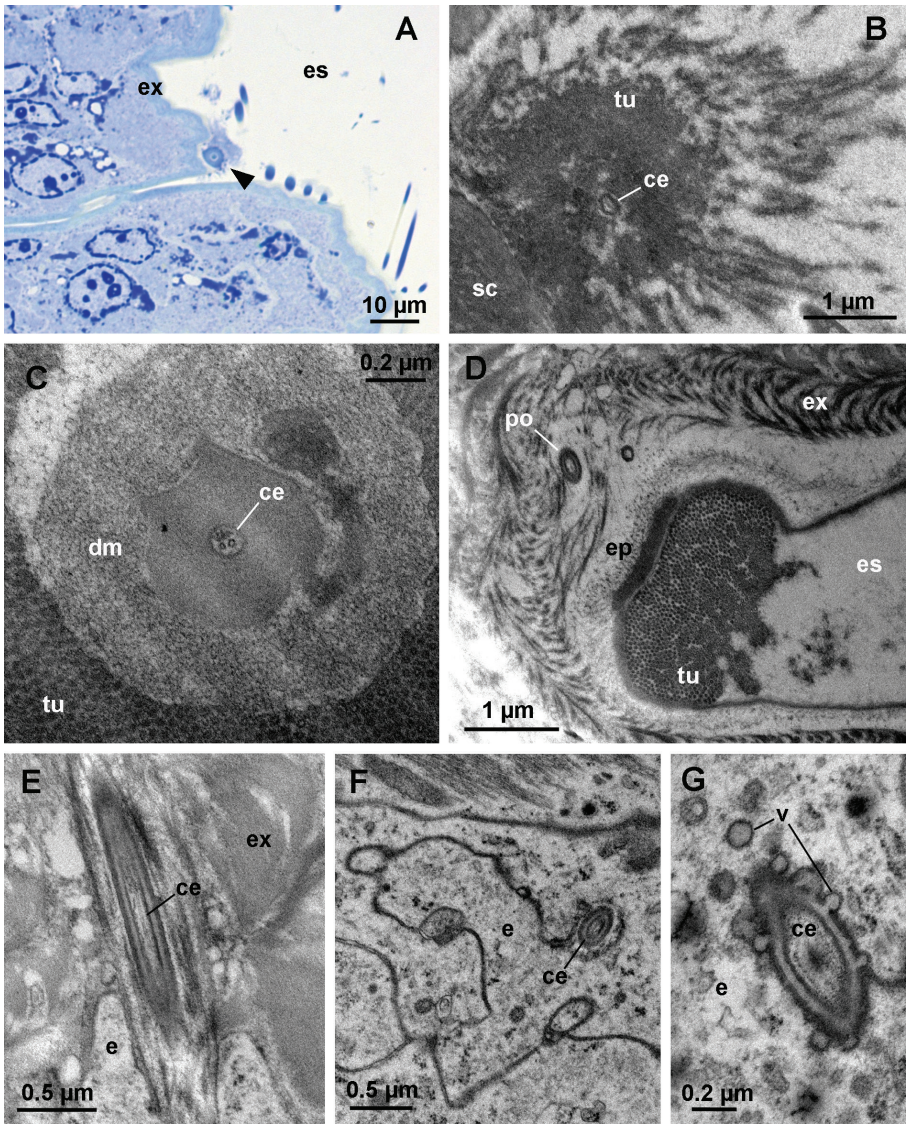

Figure 5. Cellular extensions and tubules in the ecdysial space. **A** oblique semithin section through the dorsal surface of an anterior tergite in intramolt. A bundle of tubules (arrowhead) on the surface of the new exocuticle (**ex**) is seen in cross-section. **B** electron micrograph of a bundle of tubules (**tu**). Proximally, the tubules are very densely arranged and they dissociate distally. A cellular extension (**ce**) within an electron dense sheath is located at the center of the bundle. **C** cross-section through a bundle of tubules. The cellular extension (**ce**) at the center of the bundle contains microtubules. Dense material (**dm**) surrounds the extension. **D** oblique section through the base of a bundle in intramolt. A pore (**po**) in the new cuticle is located beneath the bundle. Tubules (**tu**) protrude from the surface of the epicuticle (**ep**). **E** longitudinal section through a pore beneath a bundle of tubules. The pore contains a cellular extension (**ce**). **F** section though the epidermis beneath a bundle of tubules. A cellular extension (**ce**), enclosed in a sheath, is located in an invagination of an epidermal cell (**e**). **G** section through the cellular extension at the level of the epidermis. Vesicles (**v**) are present in the cytoplasm of the epidermal cell and are fused with the plasma membrane in proximity of the cellular extension. **es** ecdysial space, **sc** scale

tron dense sheath (Fig. 5C) and passes through the newly formed cuticle via a narrow pore (Fig. 5D, E). At the level of the epidermis, the cellular extension is enveloped by an epithelial cell (Fig. 5F). Small vesicles are present in the cytoplasm of the epidermal cell in the proximity of the extension. The membrane of some vesicles is continuous with the plasma membrane of the epithelial cell surrounding the extension (Fig. 5G).

A schematic representation of a tubular bundle surrounding a cellular extension and associated structures in late premolt is provided in Fig. 6.

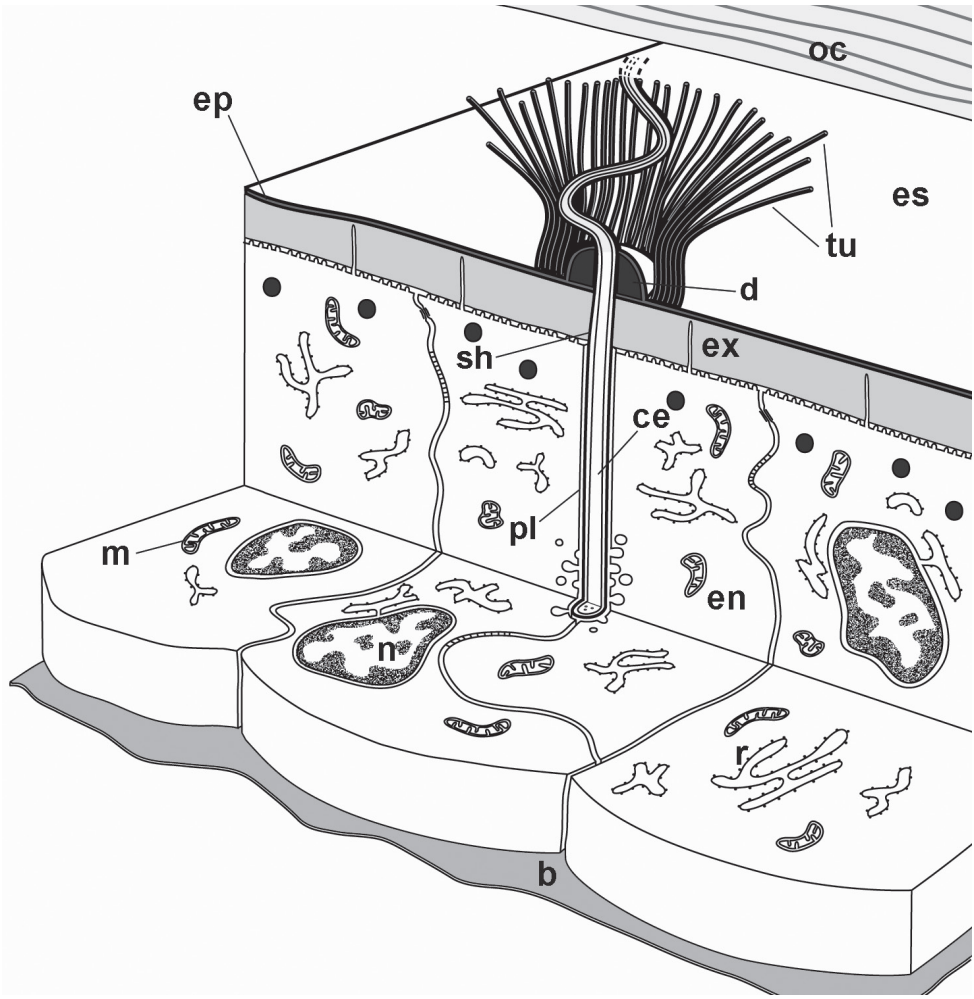

Figure 6. A schematic representation of cellular extensions associated with tubular bundles in the ecdysial space. A bundle of tubules (**tu**) surrounds a cellular extension (**ce**) that reaches into the ecdysial space (**es**). The cellular extension is enclosed in an electron dense sheath (**sh**). At the level of the epidermis, the cellular extension is surrounded by an enveloping epidermal cell (**en**). **b** basal lamina, **d** epicuticular thickening, **ep** new epicuticle, **ex** new exocuticle, **m** mitochondrion, **n** nucleus, **oc** old cuticle, **pl** plasma membrane of the enveloping epidermal cell, **r** RER.

Postmolt

During the first few days after anterior molt, rapid deposition of endocuticular lamellae takes place in anterior tergites (Fig. 7A, B). Small electron dense vesicles are no longer visible in the apical cytoplasm, but the RER remains well developed (Fig. 7B). The apical plasma membrane of epidermal cells still forms short protrusions with electron dense tips (Fig. 7B) and finger-like extensions may also be present (Fig. 7A). The cytoplasmic extensions in pore canals are less prominent than during intramolt and the pore canals appear electron lucent (Fig. 7A). After ecdysis, bundles of tubules remain present on the surface of the cuticle, but no cellular extensions can be observed

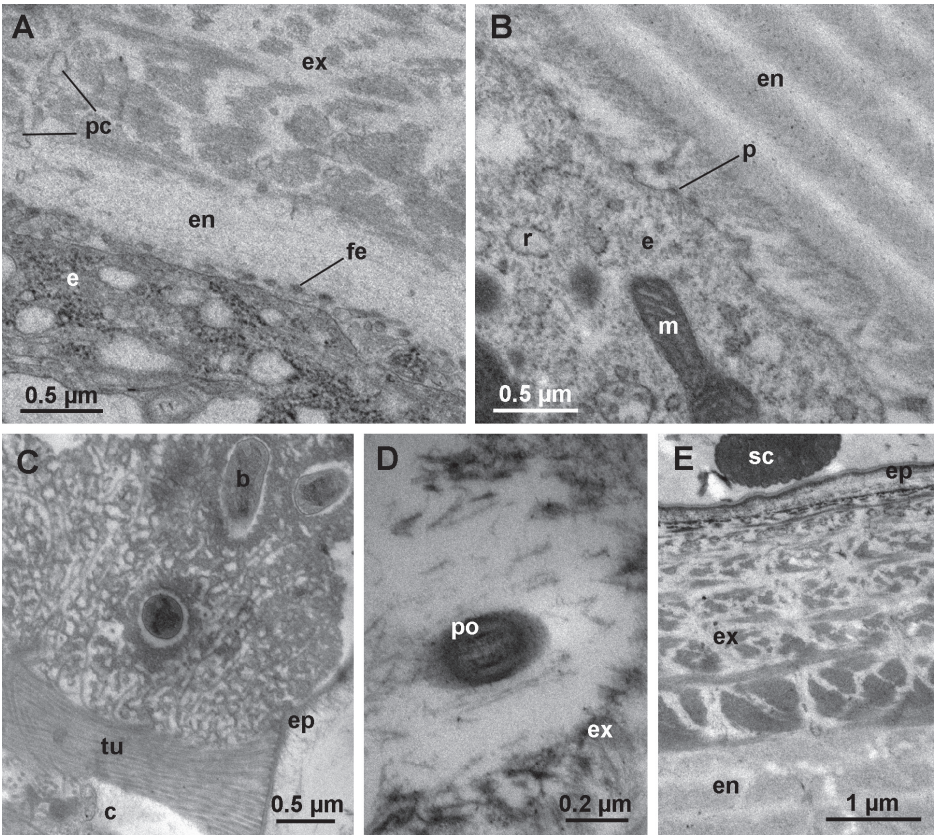

Figure 7. Ultrastructure of anterior tergites in postmolt. **A** anterior tergite shortly after molt. First lamellae of the endocuticle (**en**) are deposited proximally to the exocuticle (**ex**). Pore canals (**pc**) appear electron lucent. The apical plasma membrane of epidermal cells (**e**) forms finger-like extensions (**fe**). **B** apical region of an epidermal cell in postmolt. The epidermal cell (**e**) contains a well developed RER (**r**). The apical plasma membrane forms short protrusions (**p**) with dense tips. Several lamellae of the endocuticle (**en**) are deposited. **C** bundle of tubules (**tu**) protruding from the epicuticle (**ep**) of a tergite in postmolt. The center of the bundle (**c**) is electron lucent. **D** section through a pore (**po**) in the exocuticle beneath a bundle of tubules in postmolt. The lumen of the pore is electron dense. **E** The epicuticle (**ep**), exocuticle (**ex**) and endocuticle (**en**) in intermolt. **b** bacterium, **m** mitochondrion, **sc** scale.

in their proximity (Fig. 7C). The pores enabling the ensheathed cellular extensions at the centers of bundles to pass through the new cuticle in late premolt are observable in the cuticle in postmolt, but they appear very electron dense (Fig. 7D).

The fully synthesized tergal cuticle of an intermolt specimen of *T. albus* is presented in Figure 7E.

Discussion

The duration of the molt cycle as well as the length of individual stages within the cycle showed a high degree of variability in *T. albus* individuals, even under laboratory conditions. A typical molt cycle in *T. albus* is several times longer than in epigean isopods studied to date (Zidar et al. 1998, Štrus and Blejec 2001), but it is much shorter than that of some aquatic subterranean isopods, which molt every 9–18 months (Magniez 1975). It is difficult to compare data from studies of epigean oniscids with the results of our study, as most observations of molt cycles in terrestrial isopods were performed at room temperature while we maintained the *T. albus* culture at 11°C. By observing animals at different temperatures, Steel (1980) determined the value of Q_{10} for molting frequency in *Oniscus asellus*. On the basis of his study the expected molt cycle duration in *O. asellus* at 12 °C is approximately 8 weeks. The molt cycle in *T. albus* under laboratory conditions is thus about two times longer than would be expected in a similarly sized epigean species at a temperature close to 11 °C. This difference corresponds to different rates of respiratory metabolism between non-troglobitic isopods and *T. albus*. The expected rate of O_2 consumption of *O. asellus* at 10 °C, calculated from the previously measured metabolic rates (Phillipson and Watson 1965) and the Q_{10} for O_2 consumption in *O. asellus* (Nash 1979), would be around 0.1 ml $O_2\,g^{-1}h^{-1}$, and would therefore be roughly three times higher than the measured rate of O_2 consumption in *T. albus* at 10 °C (Simčič et al. 2010). Rates of O_2 consumption at 10 °C for the amphibious isopod *Ligia italica* (Simčič et al. 2010) and the terrestrial species *Porcellio laevis* (Husain and Alikhan 1979) are similar to the value expected for *O. asellus*. Other troglobitic crustaceans are also known to have a lower metabolic rate compared to related non-troglobitic species, which is likely to be an adaptation to the nutrient-poor cave environment (Hervant et al. 1997).

The specific postures assumed by *T. albus* after the posterior and anterior molt closely resemble those described in the epigean isopod *Armadillo officinalis* (Verhoeff 1940). The lifting of the newly molted body-half from the substrate thus appears to be widespread in Oniscidea, although it has not been reported in all species studied.

Sternal deposits of *T. albus*, consisting entirely of spherules, resemble the sternal deposits described in members of the family Ligiidae (Ziegler and Miller 1997) and *T. albus* is the first species outside Ligiidae known to form deposits of this type. There is little data on sternal deposits in Synocheta, but it has been suggested that some representatives of the group employ three-layered deposits (Ziegler 2003). If this is the case, the absence of a proximal homogenous layer in the sternal deposits of *T. albus* might represent a second-

ary reduction in the complexity of sternal deposits as an adaptation to the subterranean environment or to the amphibious mode of life of this species. This is further supported by the fact that three-layered deposits occur in Tylidae (Ziegler 2003), which is most likely the sister group of all other oniscids excluding Ligiidae (Schmidt 2008).

Consummation of the shed cuticle after ecdysis occurs in other crustaceans (Greenway 1985) and other arthropods, such as insects (Mira 2000). It is known that other terrestrial isopods also consume their exuviae after molting each body-half (Messner 1965, Ziegler et al. 2007). In contrast, *T. albus* does not ingest the posterior exuviae. Cuticle consummation as means of obtaining calcium required for the mineralization of the anterior exoskeleton is likely less crucial for the molting *T. albus*, as this species possesses very large internal calcium stores in the posterior body-half which can be utilized for cuticular mineralization after molt (personal observation). Internal calcium stores are also known to be present in some other trichoniscids (Verhoeff 1926, Ziegler 2003). It has been reported that the ligiids *Ligia hawaiensis* (Ziegler et al. 2007) and *Ligia italica* (Štrus and Blejec 2001) also do not ingest the exuviae of at least one body-half. It was shown that *Ligia hawaiensis* nevertheless retains a very high percentage of body calcium during molt which may relate to its lower body calcium content when compared to fully terrestrial isopods (Ziegler et al. 2007). Since *T. albus* also has a weakly mineralized exoskeleton and lives in a moist limestone environment, it probably has a lesser need for cuticle consummation than species that must shift greater amounts of calcium to their exoskeletons over a shorter period of time without relying on environmental calcium sources.

The ultrastructural characteristics of *T. albus* epidermal cells during cuticle synthesis, such as short protrusions of the apical plasma membrane, a well developed RER and abundant mitochondria throughout cuticle deposition as well as the presence of small, dense vesicles in the apical cytoplasm during exocuticle deposition are generally similar to those described in other oniscids (Price and Holdich 1980, Ziegler 1997, Štrus and Blejec 2001). Similar epithelial features are also present during molt in other crustacean groups (Koulish and Klepal 1981) and some aspects of epithelial ultrastructure during cuticle synthesis, for example the short protrusions of the apical plasma membrane, have also been found in insects (Locke 1961, 2001). In *T. albus*, the apical plasma membrane of epidermal cells appears highly structured during deposition of the distal lamellae of the exocuticle. Finger-like extensions and membrane invaginations may be involved in the synthesis of the exocuticle, but they might also function in intensive transport processes between the ecdysial space and the haemolymph. In *T. albus*, the epicuticle in late premolt (Fig. 3F) appears very similar to the intermolt epicuticle (Fig. 5E), indicating that there are little or no postecdysial modifications of this cuticular sublayer. This is not surprising since the epicuticular waxy layer which is modified after molt in *O. asellus* (Compere 1990) is absent in the epicuticle of *T. albus* (Hild et al. 2009). Also, the exocuticle in *T. albus* maintains its lamellar appearance after ecdysis and the premolt exocuticle greatly resembles the intermolt exocuticle (compare Figs 3G and 5E). The exocuticle in this species is therefore not deformed by the deposition of the endocuticle, as reported in *O. asellus* (Price and Holdich 1980).

The tubular structures reaching into the ecdysial space from the epicuticle in late premolt appear identical to those known from *Ligidium hypnorum* (Glötzner and Ziegler 2000) and *Ligia exotica* (Štrus et al. 2003). Tubules extending into the ecdysial space from the surface of the epicuticle therefore occur outside Ligiidae as well. In *T. albus*, they are very pronounced and associate with cellular extensions reaching through the newly formed cuticle into the ecdysial space. It has been suggested that tubules within the ecdysial space of isopods may function in water retention (Glötzner and Ziegler 2000). In the case of *T. albus*, their function is probably linked to cellular extensions reaching into the ecdysial space. The ensheathed, microtubule containing cellular extensions at the centers of tubular bundles resemble dendrites innervating isopod sensilla (cf. Crouau 1994), but their function remains to be established. The apparent absence of cellular extensions above the level of the new cuticle in proximity of the tubules in postmolt indicates that these are transient projections that degenerate after molt. The small vesicles associated with the cellular extensions in the epidermis suggest that intensive resorption and/or secretion of material may take place around the cell extensions at the level of the epidermis.

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RESEARCH ARTICLE

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Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans

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Abstract

Specialized mechanical connection between exoskeleton and underlying muscles in arthropods is a complex network of interconnected matrix constituents, junctions and associated cytoskeletal elements, which provides prominent mechanical attachment of the epidermis to the cuticle and transmits muscle tensions to the exoskeleton. This linkage involves anchoring of the complex extracellular matrix composing the cuticle to the apical membrane of tendon cells and linking of tendon cells to muscles basally. The ultrastructural arhitecture of these attachment complexes during molting is an important issue in relation to integument integrity maintenance in the course of cuticle replacement and in relation to movement ability. The aim of this work was to determine the ultrastructural organization of exoskeleton – muscles attachment complexes in the molting terrestrial isopod crustaceans, in the stage when integumental epithelium is covered by both, the newly forming cuticle and the old detached cuticle. We show that the old exoskeleton is extensively mechanically connected to the underlying epithelium in the regions of muscle attachment sites by massive arrays of fibers in adult premolt *Ligia italica* and in prehatching embryos and premolt marsupial mancas of *Porcellio scaber*. Fibers expand from the tendon cells, traverse the new cuticle and ecdysal space and protrude into the distal layers of the detached cuticle. They likely serve as final anchoring sites before exuviation and may be involved in animal movements in this stage. Tendon cells in the prehatching embryo and in marsupial mancas display a substantial apicobasally oriented transcellular arrays of microtubules, evidently engaged in myotendinous junctions and in apical anchoring of the cuticular matrix. The structural framework of musculoskeletal linkage is basically established in described intramarsupial developmental stages, suggesting its involvement in animal motility within the marsupium.

Keywords

Cuticle, chitin, microtubules, anchoring junctions, extracellular matrix, embryo

Introduction

The arthropod exoskeleton performs diverse functions, including mechanical support, sensing, prevention of desiccation and protection against pathogens and predators. Locomotion of these animals is based on extensive connections between exoskeleton and muscular system. The exoskeleton consists of a complex chitin-protein matrix, secreted by a single-layered epithelium. The chitin-protein matrix is either non-calcified or calcified, as in insects and crustaceans, respectively. Specialized epithelial cells, named tendon cells, are the sites of firm mechanical connections between exoskeleton and underlying tissues (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002). Apical membranes of tendon cells are anchored to the matrix of the cuticle and their basal surfaces are attached to muscle cells underneath (Fig. 1). A prominent ultrastructural characteristic of tendon cells are extensive bundles of microtubules, that stretch between the apical cell membrane facing the cuticle and the basal membrane engaged in the myotendinous junction. Thus, the muscle is mechanically linked to epidermis and cuticle by a complex network of cytoskeletal and junctional elements. In addition to their essential role in animal locomotion, muscle cells are expected to play important role at molting, as force-generators for movements that facilitate the exuviation.

The ultrastructure, molecular composition and differentiation of specialized anchoring complexes between exoskeleton, tendon cells and muscle cells were extensively studied in an insect *Drosophila melanogaster*, with emphasis on myotendinous junction characterization (Volk 1999, Brown 2000, Volk 2006, Schweitzer et al. 2010). Apart from *Drosophila*, there is limited data available on the fine structure and constitutive elements of exoskeleton - muscle attachment sites in other arthropods. Studies on the ultrastructural organization of connections between exoskeleton and muscles in the phases of new cuticle formation were performed in some insect species (Lai-Fook 1967, Caveney 1969, Lai-Fook and Beaton 1998) and in two groups of crustaceans: in *Euphausia superba* (Crustacea: Euphausiacea) by Buchholz and Buchholz (1989) and in podocopid ostracods by Yamada and Keyser (2009). Premolt is the unique stage preceding exuviation in which the integumental epithelium is covered by both, the newly forming cuticle apposed to the epithelial cells and the old detached cuticle above the ecdysal space. The key features of this stage in crustaceans are simultaneous disintegration of the old cuticular matrix and new cuticle formation, including secretion and recycling of organic constituents, massive calcium fluxes and modification of epithelial cells shape, size and ultrastructure (Roer and Dillaman 1993, Ziegler 1997, Compere et al. 1998, Štrus and Blejec 2001, Luquet and Marin 2004, Dillaman et al. 2005, Ziegler et al. 2005, Žnidaršič et al. 2010). Exoskeleton renewal inevitably involves the establishment of connections between the new cuticle and muscles. To supplement the knowledge on the ultrastructure of these connections and to address the issues of general principles vs. specializations of their architecture and reorganization related to molting, several species from different environments need to be studied in this respect. The exoskeleton renewal takes place during development and molting in adult specimens. There are no detailed ultrastructural data on exoskeleton anchoring to tendon cells and muscles in molting adults and in

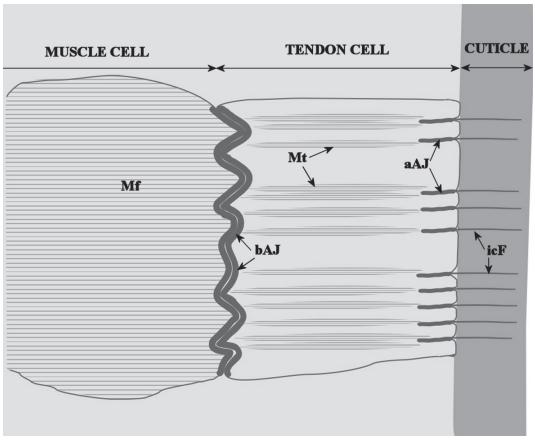

Figure 1. A scheme showing the general architecture of the muscle attachment to the epidermis in arthropods (adapted from Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002 and Subramanian et al. 2003). Specialized epithelial cells, named tendon cells, are anchored apically to the cuticle and basally to the muscle cell. **aAJ** apical adherens junction **bAJ** basal adherens junction **icF** intracuticular fibers **Mf** myofilaments **Mt** microtubules.

marsupial stages of isopod crustaceans. The embryonic development of terrestrial isopod crustaceans and hatching of embryos to marsupial mancas take place inside the female brood pouch (marsupium) and were decribed in *Porcellio scaber* from the view of overall morphology and digestive system morphogenesis, while the issue of cuticle anchoring was not addressed (Štrus et al. 2008, Wolff 2009, Milatovič et al. 2010). Fertilized eggs are released into the brood pouch, where the entire embryonic development takes place. The final phase in the embryonic development is hatching. Newly hatched animals are termed marsupial mancas and they stay inside the brood pouch for up to ten days as described in *P. scaber* females reared in the laboratory (Milatovič et al. 2010).

Here we report new data on the ultrastructural architecture of anchoring complexes comprising exoskeleton, tendon cells and muscles in adult premolt isopod crustaceans, in premolt marsupial mancas and in prehatching embryos. Our study is focused primarily to connections between the complex matrix of the exoskeleton and tendon cells, modified epithelial cells at the sites of muscles attachment. To the best of our knowledge, the exoskeleton anchoring to underlying tissues in embryos and marsupial mancas of crustaceans has not been characterized before and its ultrastructural organization is presented here. Comparative evaluation of the results with respect to the other arthropods, particularly to insect model organism *D. melanogaster,* is presented. The involvement of these anchoring connections in molting and in intramarsupial motility is discussed.

Methods

Specimens of *Ligia italica* Fabricius, 1798 (Crustacea: Isopoda) were collected at the Piran Bay coast in Slovenia. Animals were inspected for ventral sternal deposits and

premolt adult specimens were anaesthetized. The dorsal parts of pereonites were isolated, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and postfixed by 1% OsO_4. After washing and dehydration in a graded series of ethanol, samples were embedded in an epoxy resin mixture. Semithin sections were stained with Azure II. – Methylene blue. Ultrathin sections were either imaged non-contrasted or contrasted with uranyl acetate and lead citrate.

Specimens of *Porcellio scaber* Latreille, 1804 (Crustacea: Isopoda) prehatching embryos and marsupial mancas were isolated from brood pouches of females maintained in laboratory culture. Determination of intramarsupial developmental stages was performed as described in Milatovič et al. (2010). Isolated embryos and mancas were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Prior to fixation, the vitelline membrane of embryos was carefully perforated with a thin needle. After washing with 0.1 M cacodylate buffer, the samples were postfixed in 1% OsO_4 for 2 hours, washed again and dehydrated in a graded series of ethanol. Specimens were embedded in Agar 100 resin. Prior to embedding, mancas were perforated with a thin needle for better infiltration of resin. Semithin sections were stained with Azure II. - Methylene blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Light microscopy was performed by AxioImager Z.1 microscope (Zeiss) equiped with an AxioCam HRc camera and Axiovision software. Ultrastructural imaging was performed by CM 100 transmission electron microscope (Phillips) equiped with a BioScan 792 digital camera (Gatan) and Digital Micrograph software.

Results

Adult premolt specimens

The ultrastructural architecture of the exoskeleton – muscles attachment regions was analysed in the dorsal parts of pereonites in adult premolt *Ligia italica*. The pre-ecdysial cuticle was tightly connected to the underlying tendon cells and muscles already in the early premolt (Fig. 2). Extensive connections were established between the chitin-protein matrix of the newly forming cuticle and the apical parts of the tendon cells in the early phase of cuticle elaboration. These matrix – cell linkages consisted of numerous fibrous structures. The most intriguing result was that the fibers traversed the entire new cuticle, spanned the whole ecdysal space and protruded deep into the old cuticle (Figs 2B, C). They extended from the tendon cell apex up to the exocuticular layer in the old cuticle. The continuity of these fibers was clearly followed in some sections, revealing a direct mechanical connection of the detached exoskeleton to epithelium in these regions. Fibrous structures were arranged in parallel to one another and in the same direction as microtubules arrays in the tendon cells and myofilaments arrays in the muscle cells. This direction is roughly perpendicular to the body surface. Fibrous connections followed approximately straight lines and did not display any branching or prominent curvatures. General architecture of the preecdysal cuticle at muscle

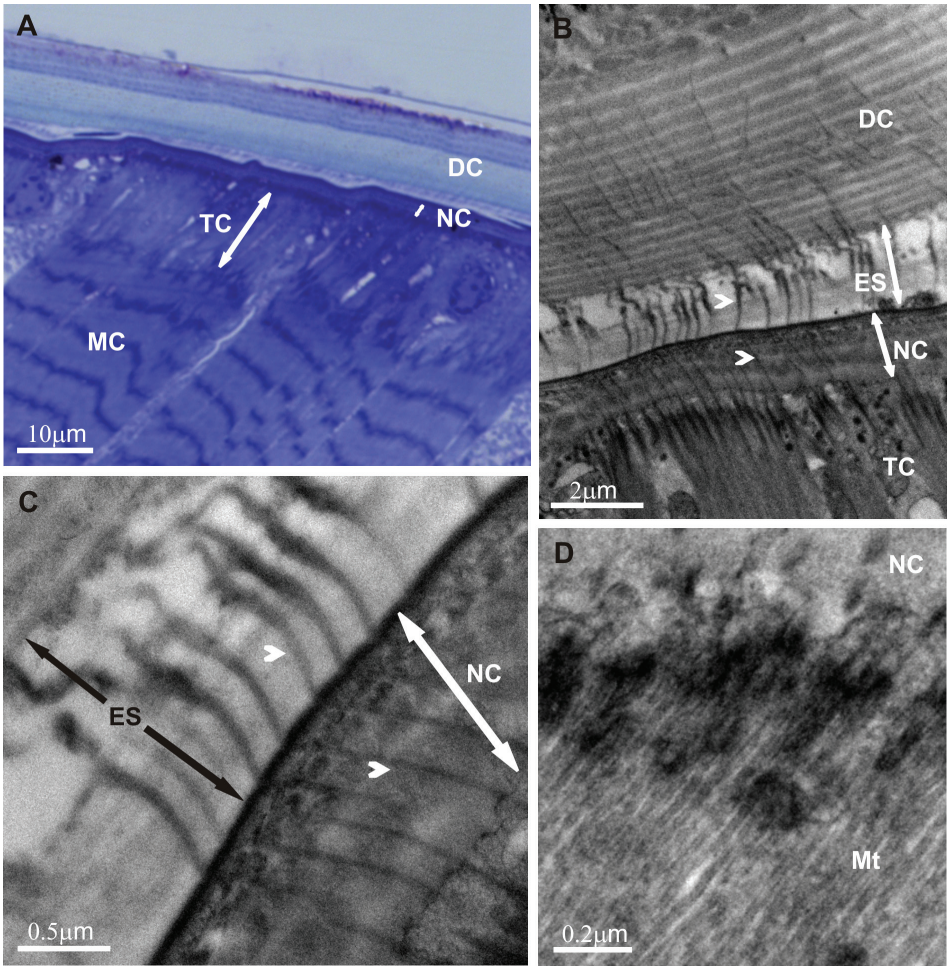

Figure 2. Exoskeleton – muscle attachment in the dorsal parts of pereonites in adult premolt *Ligia italica*. Overview of the muscle attachment in a specimen with a detached and a newly forming cuticle (**2A** a semithin section). The new cuticle is extensively connected to the underlying tendon cells already in the early premolt. Fibrous connections running from the apical region of tendon cells through the new pre-ecdysal cuticle and ecdysal space up to the exocuticle of the detached exoskeleton are evident **(2B, C)**. Parallel arrays of microtubules and apical electron dense plaques are characteristic for tendon cells **(2D)**. **DC** detached cuticle **NC** new cuticle **ES** ecdysal space **MC** muscle cell **TC** tendon cell **Mt** microtubules; arrowheads – fibrous connections.

attachment sites was similar to that in the other regions, displaying epicuticular and exocuticular layers and characteristic pattern of chitin-protein fibers arrangement.

Extensive parallel arrays of microtubules inside the tendon cells were aligned in the apical to basal direction (Fig. 2D). A concourse of microtubules towards the cytoplasmic densities at the apical membrane (hemidesmosome-like structures) of tendon cells was evident. On the basal side the microtubules were positioned close to the electron dense plaques along the basal membrane, engaged in myotendinous junction.

Prominent anchoring junctions were evident between muscle cells and tendon cells (Fig. 3). The entire basal membrane of the tendon cell was intensely folded in a zigzag pattern, exactly matching the folding of the muscle cell sarcolemma beneath. Both cell surfaces contributing to this junction were closely apposed and the intervening layer of the extracellular matrix material was not conspicous. The complex connection between these two cells in premolt specimens corresponds to characteristic design in animals with one complete cuticle (Supplementary figure), comprising electron dense plaques beneath both cell membranes and extracellular material in the narrow intercellular space.

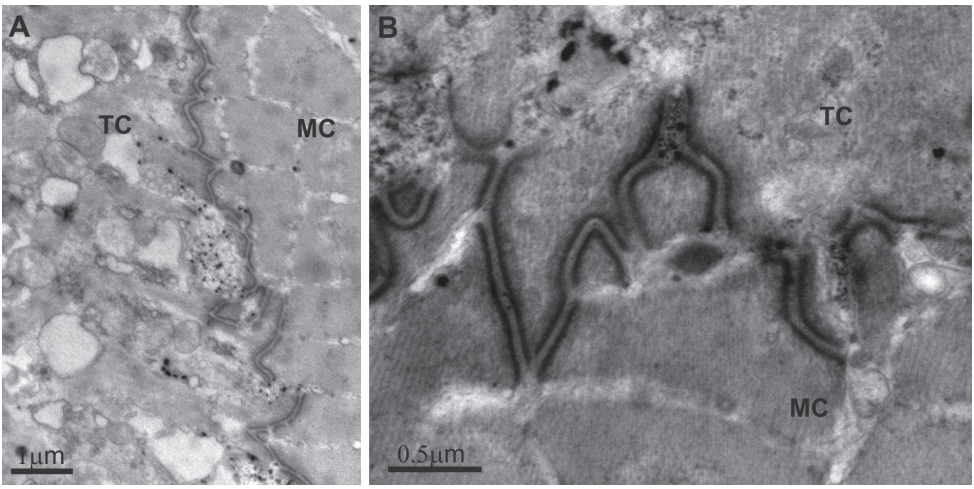

Figure 3. Myotendinous junction in the dorsal parts of pereonites of premolt adult *Ligia italica* is an extensive anchoring junction. The entire zigzag folded basal and apical membranes of tendon and muscle cells, respectively, are engaged in this outstanding intercellular mechanical connection **(3A)**. Prominent electron dense cytoplasmic plaques are evident along both cell membranes, separated by a thin layer of extracellular matrix **(3B)**. **MC** muscle cell **TC** tendon cell.

Intramarsupial premolt animals

Development of *Porcellio scaber* embryos and marsupial mancas involves renewal of the exoskeleton. Prehatching embryo and several marsupial mancas displaying morphological attributes of premolt were analysed in this study. The detachment of the old cuticle and disintegration of the basal parts of the detached cuticle were identified in the integument of these specimens. In addition, the substantial new cuticle formation was evident in marsupial mancas. The exoskeleton in the prehatching embryo and in the premolt marsupial mancas was extensively connected to tendon cells and muscles underneath and extensive intercellular junctions between muscle cells and tendon cells were established (Fig. 4).

Numerous fibrous connections between the detached cuticle and the apical membrane of tendon cells were evident in all premolt intramarsupial animals examined (Fig. 5). The newly forming cuticle in marsupial mancas, consisting of epicuticle and a few layers of the pre-ecdysal procuticle, was mechanically connected to tendon cells.

Tendon cells ultrastructurally resembled the adult arthropod tendon cells and were characterized by apicobasal arrays of microtubules. Apically, the microtubules were found close to oblong electron dense regions (hemidesmosome-like structures), aligned in the same direction (Figs 5D–F and Fig. 6). In longitudinal and in oblique sections the profiles of microtubules were evidenced in close proximity to the electron dense plaques of junctions along the tendon cell basal membrane in both, prehatching embryos (Figs 7A, B) and marsupial mancas (Figs 7C, D).

Myotendinous junctions displayed a characteristic zigzag outline, occupying the entire tendon – muscle interface (Fig. 7). From the structural point of view, the myotendinous junctions in marsupial mancas closely resembled these junctions in adult

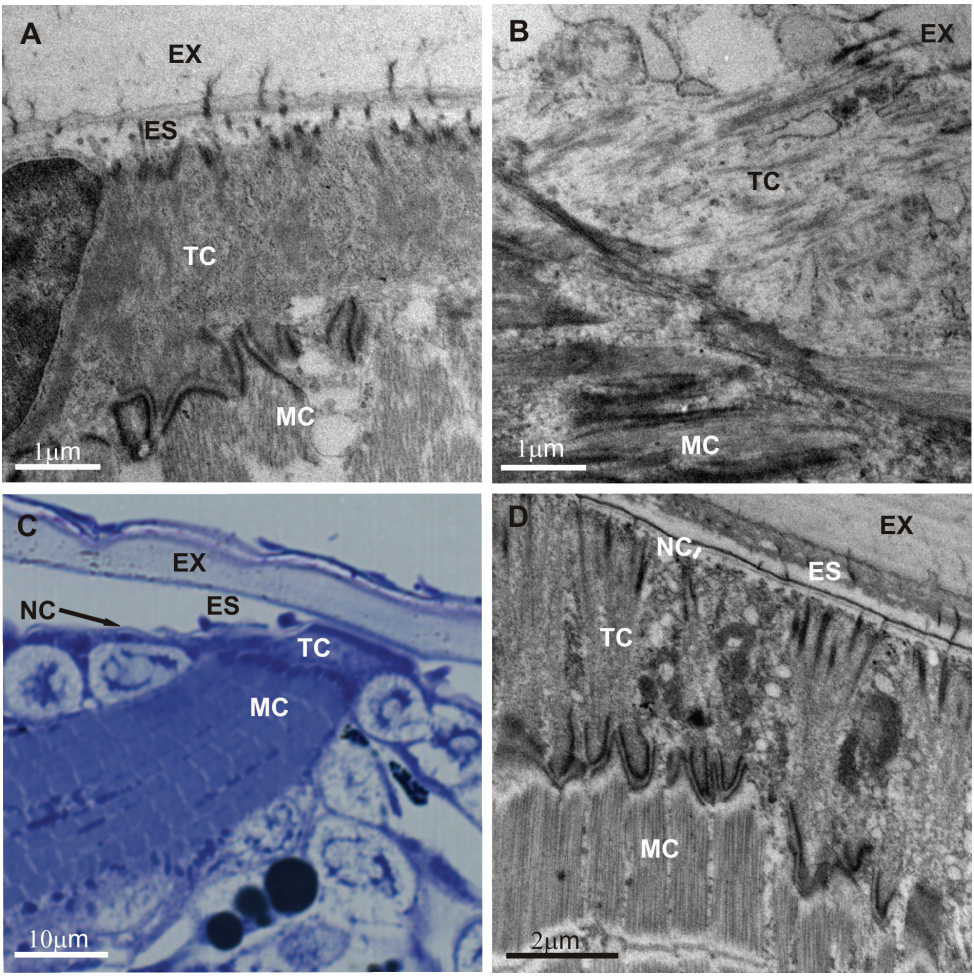

Figure 4. Overview of exoskeleton anchoring to tendon cells and muscles in intramarsupial developmental stages of *Porcellio scaber*. In prehatching embryos **(4A, B)** and in premolt marsupial mancas (**4C** a semithin section and **4D**) the connections between the exoskeleton, tendon cells and muscle cells were already established. **EX** exoskeleton **ES** ecdysal space **NC** new cuticle **TC** tendon cell **MC** muscle cell.

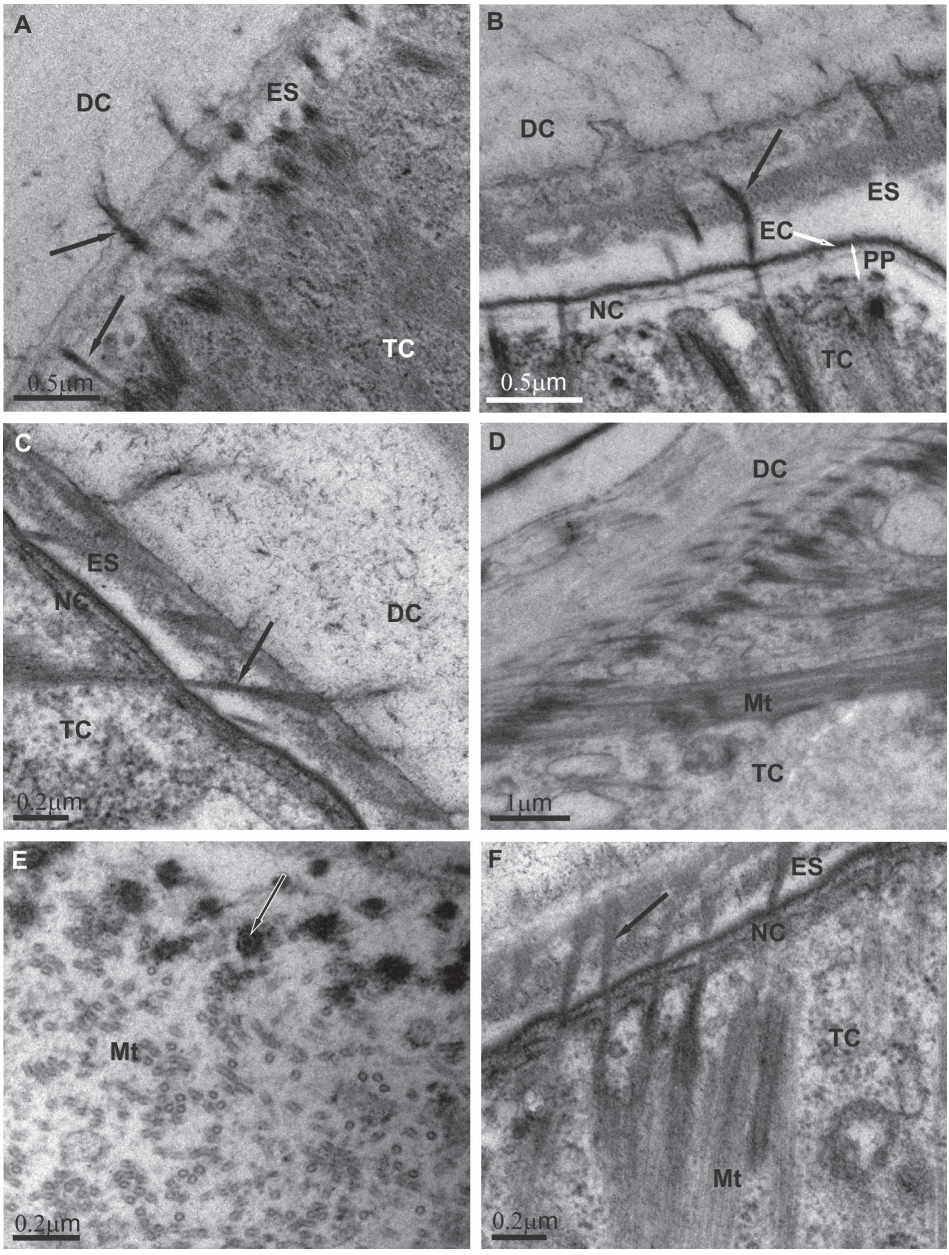

Figure 5. Anchoring junctions between the detached cuticle and the apical part of tendon cells in the prehatching embryo **(5A, D, E)** and marsupial mancas **(5B, C, F)** of *Porcellio scaber* (ultrathin cross-sections). The newly forming cuticle in marsupial mancas, consisting of the epicuticle and pre-ecdysal procuticle, was mechanically connected to tendon cells. Numerous bundles of fibers (arrows) running from tendon cells through the new cuticle and ecdysal space into the detached cuticle are evident. Microtubules were found in close proximity to electron dense plaques at the apical surface of tendon cells. **DC** detached cuticle **NC** new cuticle **EC** epicuticle **ES** ecdysal space **PP** pre-ecdysal procuticle **TC** tendon cell **Mt** microtubules.

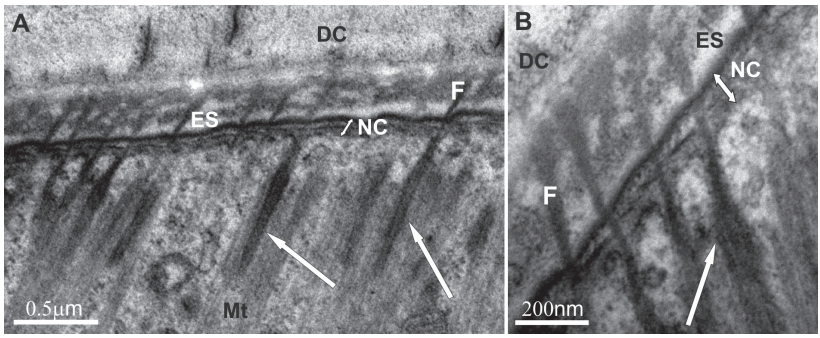

Figure 6. Electron dense plaques (hemidesmosome-like structures) at the apical surface of the tendon cells in the marsupial manca of *Porcellio scaber*. Electron dense plaques (arrows) are associated with microtubules **(Mt)** in the cytoplasm of the tendon cell and with the bundles of fibers **(F)** running through the ecdysal space **(ES)** on the opposite side. **DC** detached cuticle **NC** new cuticle.

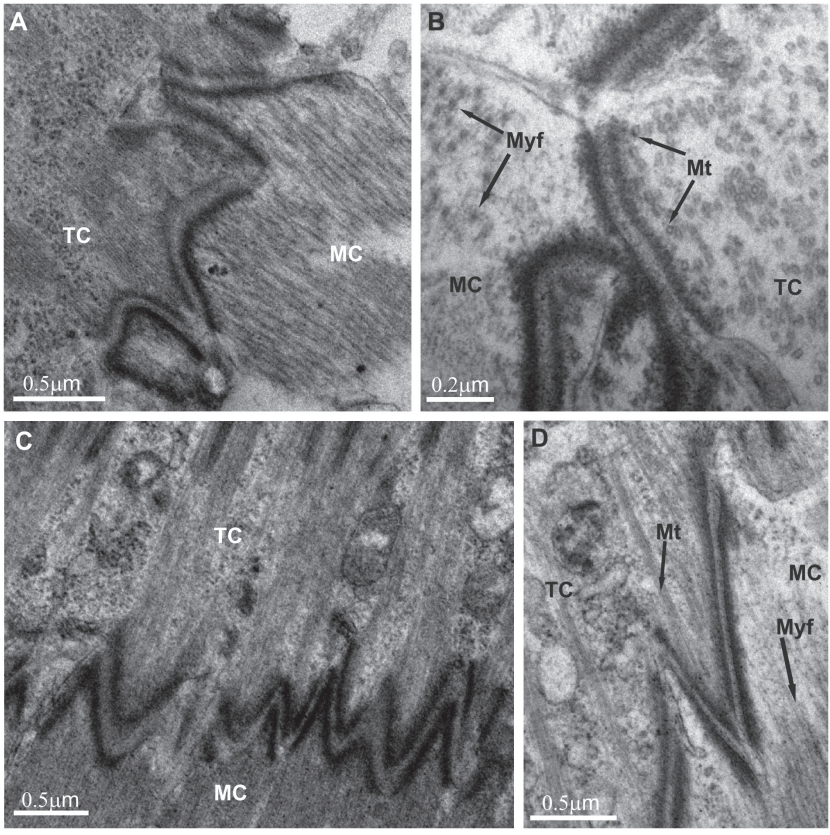

Figure 7. Myotendinous junction in the *Porcellio scaber* prehatching embryo **(7A, B)** and marsupial mancas **(7C, D)**. The cytoplasmic plaque of the anchoring junction at the basal membrane of tendon cell is more lucent and thinner than the accompanying plaque at the apical membrane of the muscle cell in the prehatching embryo **(7A, B)**. Microtubules of the tendon cells are in close proximity to the basal dense plaques. **TC** tendon cell **MC** muscle cell **Mt** microtubules **Myf** myofilaments.

arthropods, comprising a thin layer of extracellular material between cell membranes and dense cytoplasmic plaques of approximately equal densities and thicknesses below the both cell membranes (Figs 7C, D). On the other hand, in the prehatching embryo, the intracellular membrane-associated layer of dense cytoplasmic material contributing to the anchoring junction in a tendon cell was more lucent and thinner than that contributing to the junction in a muscle cell (Figs 7A, B).

Discussion

Specialized anchoring complexes between exoskeleton, tendon cells and force-generating muscle cells are essential features of the musculoskeletal system in arthropods (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998, Bitsch and Bitsch 2002). A complex network of cytoskeletal and junctional elements constitutes the main scaffold of specialized mechanical connections between the calcified exoskeleton and underlying tissues in crustaceans (Mellon 1992). These elaborate connections between different types of cells and hierarchically structured extracellular matrix constitute a common functional network, enabling animal movements, and are thus involved also in animal responses to various physiological and environmental cues.

Our results show that the old exoskeleton is still mechanically attached to the underlying epithelium in the regions of muscle attachment sites for a certain period of the premolt phase in isopod crustaceans. We have observed massive arrays of fibers running from tendon cells through the new cuticle and ecdysal space up to the distal layers of the detached cuticle in adult premolt *L. italica* and in premolt intramarsupial specimens of *P. scaber*. The continuity of these fibers was clearly evidenced in both species. The fibers extending from the tendon cells deep into the cuticular matrix in non-molting specimens are known from previous studies of arthropods (Noble-Nesbitt 1963, Mellon 1992, Lai-Fook and Beaton 1998) and were also reported in some studies of molting insect species (Lai-Fook 1967, Caveney 1969, Lai-Fook and Beaton 1998) and in two studies of molting crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009).Different authors use different names to designate these fibers, which is rather confusing. They are termed muscle attachment fibers (Caveney 1969), tonofibrillae (Buchholz and Buchholz 1989, Lai-Fook and Beaton 1998, Tucker et al. 2004), tonofilaments (Subramanian et al. 2003), intracuticular fibers (Mellon 1992) and intracuticular rods (Criel et al. 2005). To the best of our knowledge, the macromolecules constituting these fibers have not been identified yet, neither in insects, nor in crustaceans. They are described as dense strands of cuticular material that project into the apical invaginations of tendon cells (Tucker et al. 2004). In contrast to comprehensively resolved molecular architecture of the tendon – muscle junction in *Drosophila* (Volk 2006, Schweitzer et al. 2010), the proteins that connect the apical surface of the epidermis to the cuticle are not known (Brown 2000). The ultrastructural organization of the fibrous connections between the detached cuticle and tendon cells described in our study for two isopod species closely resembles that reported for the

premolt *Euphausia superba* by Buchholz and Buchholz (1989) and for the premolt podocopid ostracods by Yamada and Keyser (2009). As in *E. superba* and in ostracods the new preecdysal cuticle in isopods is secreted while fibers maintain the connection between the old cuticle and epidermis. Yamada and Keyser (2009) in addition described the formation of the new cuticle in detail. They reported that the new epicuticle was deposited around the extended intracuticular fibers and suggested that the intracuticular fibers increase their length before the deposition of the new epicuticular material. The continuity of the fibers stretching from the tendon cells through the new cuticle and ecdysal space to the old cuticle is clearly evident in our images of premolt isopod integument. This indicates that the structural reorganization of the existing fibers occurs during premolt rather than extensive formation of the new fibers. This is also in agreement with previous reports on the continuity of the tonobrillae in molting insects (Lai-Fook 1967) and crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009). We consider that cuticle-muscle attachment complexes supposedly helping or enabling movements during shedding of the old cuticle are also involved in maintenance of integument integrity of the 'two-cuticle' stage in the premolt isopods. Fibers connecting the detached cuticle to the underlying tissues likely serve as final anchoring sites before exuviation. These anchoring connections to the underlying epidermis and muscles may also be involved in limited locomotory activity of the animal during molting. As the newly forming cuticle underneath is already extensively connected by such fibers to the apical membrane of tendon cells and indirectly to muscles below, at least elementary locomotion may thus be enabled also immediately after exuviation.

Apicobasally oriented microtubule arrays are formed in several types of polarized epithelial cells. Tucker et al. (2004) report that most microtubules in *Drosophila* tendon cells exhibit unusually large diameters of up to 30 nm, while in our study the usual microtubules of 24 nm in diameter were observed in the tendon cells of adult and intramarsupial isopods. Here we show that tendon cells in prehatching embryos and marsupial mancas of *P. scaber* already include a substantial apicobasally oriented transcellular arrays of microtubules, evidently engaged in myotendinous junctions and in apical anchoring of the cuticular matrix. Thus we consider that in the prehatching embryos and marsupial mancas the structural framework of musculoskeletal linkage is basically established and could assist in animal motions within the marsupium. This consideration is further supported by our observation that marsupial mancas display pronouced body movements inside the marsupial fluid and is supported also by the study of Milatovič et al. (2010), who reported that embryo hatching from the vitelline membrane involves swelling and active movement.

The myotendinous junction in the dorsal parts of pereonites in molting *L. italica* is a zigzag patterned junction of the tendon cell basal membrane and muscle cell sarcolemma, with an inconspicious layer of extracellular matrix inbetween. The entire basal surface and apical surface of tendon and muscle cell, respectively, contribute to this heterotypic adherens junction. Both interacting cell membranes are extensively folded, which increases the surface area of contact and contributes to enhanced mechanical resistance. The myotendinous connection in molting *L. italica* structurally resembles

that described in non-molting specimens and in *Drosophila*. The myotendinous junction in *Drosophila* is considered to be composed of two sets of hemiadherens junctions with an intervening layer of extracellular matrix material that has a substantial thickness in certain situations (Prokop et al. 1998, Tucker et al., 2004). In recent years the molecular machinery involved in the formation of these junctions has been increasingly elucidated. The *Drosophila* myotendinous connection is integrin dependant and involves transmembrane integrins that connect to protein ligands in the extracellular matrix and to the cytoskeleton inside the cell (Prokop et al. 1998, Delon and Brown 2008). This strategy is implemented in both, direct and indirect muscle attachments in *Drosophila*. The molecular composition of tendon cell – muscle cell junction has not been resolved in crustaceans, but similar principles involving integrins and associated linking proteins are expected to be implied.

The ultrastructural arhitecture of myotendinous junctions in the prehatching embryos and marsupial mancas of *P. scaber* analysed in this study is similar to the general structural outline of adult arthropod muscle attachments. In marsupial mancas it appears to be structurally fully elaborated, while in the prehatching embryo it may not be completely formed. The cytoplasmic plaques engaged in anchoring junctions at the basal membrane of the tendon cell in the prehatching embryo are markedly electron lucent and thinner as compared to the opposing cytoplasmic plaques in the muscle cells, while in adult arthropods the myotendinous anchoring junctions comprise cytoplasmic plaques of similar thicknesses and densities in both cells. A similar situation was observed in *Drosophila* in vitro culture of primary embryonic cells by Tucker et al. (2004), who reported that differentiating tendon cells are characterized also by thinner hemiadherens junction as compared to the associated muscle cell. Thus we consider that this structural feature may indicate the not completely differentiated junction. The functional significance of this is not yet clear.

Conclusions

Cell to cell and cell to matrix anchoring junctions, together with their associated cytoskeletal elements, are engaged in providing tissue structural scaffold and integrity, but more than that, they are increasingly discussed from the perspective of tissue and cell dynamics (Zaidel-Bar et al. 2010). These junctions undergo dynamic changes during development and during regeneration in adulthood. The overall general architecture of the exoskeleton-muscle attachment in isopod crustaceans described in our study is similar to that reported for other arthropods. We show here that elaborate anchoring junctions between the tendon cell apical membrane and the extracellular matrix provide attachment of the exoskeleton to the underlying tissues also during cuticle replacement in adult and in intramarsupial developmental stages of isopod crustaceans. Thus they contribute to integument integrity during molting and together with associ-

ated microtubules in tendon cells and myotendinous connections likely enable at least basic movements in this period. As cuticle replacement involves old cuticle detachment followed by the new chitin – protein matrix secretion at the apical membranes of epidermal cells, the rearrangement and remodeling of anchoring junctions between the tendon cells and cuticle are expected. The continuity of the fibers ranging from the tendon cells through the new cuticle and ecdysal space to the old cuticle was evident in premolt adult and intramarsupial isopods. This indicates that during premolt the reorganization of fibers and their associations with the cuticle takes place, rather then extensive formation of the new fibers. Our considerations are in agreement with previous reports on the continuity of tonofibrillae, which maintain the connection between the tendon cells and the old cuticle in molting insects (Lai-Fook 1967) and crustaceans (Buchholz and Buchholz 1989, Yamada and Keyser 2009).

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Supplementary figure

Exoskeleton-muscle attachment in the adult Ligia italica with the entire cuticle of the usual thickness. (doi: 10.3897/zookeys.176.2445.app) File format: Portable (Public) Network Graphic (png).

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Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial mancas

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Abstract

An important adaptation to land habitats in terrestrial isopod crustaceans is development of embryos in a fluid-filled female brood pouch, marsupium. The study brings insight into the structure and protective role of egg envelopes and cuticle renewal during ontogenetic development of *Porcellio* embryos and marsupial mancas. Egg envelopes cover embryos, the outer chorion until late-stage embryo and the inner vitelline membrane throughout the whole embryonic development. Egg envelopes of *Porcellio* have relatively simple ultrastuctural architecture compared to *Drosophila* egg envelopes. Exoskeletal cuticle is produced in late embryonic development by hypodermal cells of the embryo and is renewed in further development in relation to growth of developing embryos and mancas. Cuticle structure and renewal in prehatching late-stage embryos and marsupial mancas exhibit main features of cuticle in adults. Epicuticle is thin and homogenous. The characteristic arrangement of chitin-protein fibers and the dense distal layer in exocuticle are hardly discernible in prehatching embryo and distinct in marsupial mancas. Endocuticle consists of alternating electron dense and electron lucent sublayers and is perforated by pore canals in both stages. Differences from adult cuticle are evident in cuticle thickness, ultrastructure and mineralization. Signs of cuticle renewal in prehatching embryo and marsupial mancas such as detachment of cuticle from hypodermis, partial disintegration of endocuticle and assembly of new cuticle are described.

Keywords

Chorion, vitelline membrane, cuticle, molting, ontogenetic development, terrestrial isopods

Introduction

The unique feature of embryonic development in isopod and amphipod crustaceans (Peracarida) is its location in the brood pouch on the ventral side of female body (marsupium). The marsupium has likely been of a great adaptive significance in the colonization of land by crustacean species as it allows embryonic development to occur in aqueous environment within the protected chamber (Hornung 2011, Warburg 2011). Two main types of marsupium are distinguished in Oniscidea, the amphibian or open type and the terrestrial or closed type marsupium (Hoese and Janssen 1989). In the former, marsupium is partly open and water from the external environment can pass into the marsupium. In the closed type, marsupium is a watertight structure, provisioned only with fluid from the mother. Marsupial fluid is persistently osmotic and ionic regulated, probably via transporting epithelia of segmental cotyledons, which hang down from the overlying sternites (Surbida and Wright 2001). Depending on the level of maternal control of the marsupial environment, osmotic tolerance of the embryos and marsupial mancas is of adaptive importance. Protective envelopes between embryo/manca and marsupial fluid function against potential physiological stresses, including osmotic and ionic variation and desiccation in marsupial environment (Charmantier and Charmantier-Daures 2001, Surbida and Wright 2001). Ontogenetic development of terrestrial isopod crustacean *Porcellio scaber*, from released fertilized eggs to embryos and marsupial mancas, occurs in the terrestrial type marsupium. Intramarsupial development of *P. scaber* lasts approximately 35 days (Milatovič et al. 2010), and during development individuals in different stages are coated by different protective envelopes - egg envelopes (chorion and vitelline membrane) and cuticle. During growth of embryos and mancas egg envelopes are shed and cuticle is renewed.

Two egg envelopes, the outer chorion and the inner vitelline membrane, are produced during oogenesis by the somatic follicle cells of the female reproductive system and cover the embryo until the transition to the late-stage embryo and hatching, respectively. Arthropods evolved egg envelopes of different morphologies and complexities as a consequence of embryonic development in different environments. The data on egg envelopes (chorion and vitelline membrane) structure derive mainly from the studies on the fruit fly *Drosophila melanogaster*, as this model species offers wide opportunities for genetic studies (Margaritis et al. 1980, Jagadeeshan and Singh 2007), while there are no data on isopod chorion and vitelline membrane ultrastructure. In contrast to *Porcellio scaber* embryonic development in the female protected environment, *Drosophila* embryonic development takes place independently of the female. The egg envelopes of *Drosophila*, especially the chorion, are structurally complex and their proteins show signs of evolving under selection by ecological factors. At the morphological level, differences in egg envelopes surfaces between specialists and generalists of *Drosophila* species were found, particularly in the surface ridges and surface porosity (Jagadeeshan and Singh 2007).

Cuticle is the innermost protective layer, formed later during intramarsupial development and is produced by hypodermal cells of the embryo. In adult arthropods the

cuticle is a complex hierarchically structured extracellular matrix, consisting of chitin, proteins and lipids, hardened mostly by mineralization in crustaceans in contrast to insect cuticle which is only sclerotized. It comprises the distal epicuticle, the exocuticle in the middle and the proximal endocuticle. Several reports were published on cuticle structure in adult isopods, mostly in *Porcellio scaber* (Price and Holdich 1980, Štrus and Compere 1996, Ziegler 1997, Glötzner and Ziegler 2000, Štrus and Blejec 2001, Hild et al. 2008, Hild et al. 2009). The thin and non-calcified epicuticle is composed mainly of lipoproteins and consists of thinner 5-layered outer epicuticle and thicker inner epicuticle. The exocuticle, comprising sublayers of chitin–protein fibers arranged in characteristic pattern and the endocuticle, consisting of lamellar chitin–protein sublayers, are calcified. In addition, the thin non-calcified membranous layer lies between the endocuticle and the epithelial cells. The ultrastructure and composition of the cuticle in isopod embryos and marsupial mancas have not been studied in detail, while cuticle structure is well described in *Drosophila melanogaster* embryos (Locke 2001, Payre 2004, Moussian et al. 2006, Moussian 2010). Fully formed cuticle in *Drosophila* embryo is organized in distinct horizontal layers. The distal lipoprotein epicuticle is subdivided in the outer thin epicuticle (cuticulin layer) and the inner thick epicuticle and the proximal chitin-protein procuticle consists of several lamellae.

Cuticle renewal is related to growth in arthropods. In crustaceans molting frequently recurs during adult life. Isopod crustaceans molt in two phases, separately molting posterior and anterior parts of the body. Molt cycle begins with premolt stage, when remarkable morphological changes of the integument occur. The old cuticle separates from the underlying epithelium (apolysis). Epithelial cells secrete a new cuticle, starting with the epicuticle and followed by pre-ecdysal exocuticle. The old and the new cuticles are separated by an extracellular compartment, the ecdysal space, containing different material involved in cuticle renewal. At molting the old cuticle is shed and the new cuticle is further produced, forming post-ecdysal endocuticle. Postmolt stage is marked by soft body surface with progressive hardening of the exoskeleton until the intermolt stage.

In this study we present new data on the ultrastructural architecture of egg envelopes, including chorion and vitelline membrane, and on the ultrastructural characteristics of cuticle renewal in embryos and marsupial mancas of isopod crustaceans *Porcellio scaber* and *Porcellio dilatatus*. Comparison of envelopes structure in terrestrial crustaceans and insects will bring new insights into the protective role of egg envelopes and cuticle renewal in developing embryos of these two terrestrial arthropod groups with different developmental strategies.

Methods

Animals were maintained and bred in a laboratory culture. Staging system of *P. scaber* ontogenetic development, based on morphological characteristics of embryos and marsupial mancas, was used in this study (Milatovič et al. 2010). Embryos of *P. dilatatus* were used in scanning electron microscopic studies of egg envelopes.

The embryos and marsupial mancas of different developmental stages are shown in Figures 1A, 2A, 3A, 4A and 5A-C in the Results. The term early-stage embryo is used for embryos with large amount of yolk mass in the central part and no visible limb buds. A mid-stage embryo has visible developing limb buds and two midgut glands primordia, which enclose yolk. After bending ventrally and shedding of chorion, embryos are termed late-stage embryos. Prior to hatching swelled embryo inside the vitelline membrane is described as a prehatching late-stage embryo. When late embryos hatch from the vitelline membrane they become marsupial mancas. The progress of development of *P. scaber* marsupial mancas is characterized by the following morphologically discernible modifications: reduction of the midgut glands size due to yolk consumption, increase in exoskeleton pigmentation, enlargement of body size and pronounced locomotion (Wolff 2009, Milatovič et al. 2010). In previous studies stages of marsupial mancas were not precisely determined. For this reason and according to the morphological characteristics listed above, we determined three sequential developmental stages of marsupial mancas, early-stage, mid-stage and late-stage marsupial mancas. The term early-stage manca is used for 1.5 – 1.6 mm long mancas with no or very little locomotion inside the marsupium, with scarce chromatophores on the body surface and with the midgut yolk extending into the pleon. Mid-stage mancas are 1.7 - 1.8 mm long, with darker pigmentation on the head region and tergites and with the midgut yolk only partly extending into the pleon. Late-stage mancas are 1.9 - 2.0 mm long, with pronounced locomotion of the whole body and pereopods. The yolk in the midgut extends only to the end of the pereon.

Embryos and mancas at different stages of development were isolated from the marsupium and 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 washing in cacodylate buffer, the samples were postfixed in 1% osmium tetroxide for 2 hours, washed again and dehydrated in a graded series of ethanol.

Embryos and mancas of *P. scaber* for light microscopy (LM) and transmission electron microscopy (TEM) were embedded in Agar 100 resin. Prior to embedding, mancas were perforated with a thin needle for better infiltration of resin. Semithin sections were made with a glass knife, stained with Azure II - Methylene Blue and imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRC Axiocam camera. Ultrathin sections were made with a Reichert Ultracut S ultramicrotome (Leica), contrasted with 4% uranyl acetate for 10 minutes and 10% lead citrate for 5 minutes and inspected with a Philips CM100 transmission electron microscope, equipped by BioScan 792 camera (Gatan).

After dehydration in a graded series of ethanol and in acetone, *P. dilatatus* specimens for scanning electron microscopy (SEM) were transferred into hexamethyldisilazane (HMDS) to perform chemical drying. Mounted specimens were coated with gold and observed with scanning electron microscopes (Philips XL20 and Philips XL30).

For histochemical detection of calcified tissue, mancas of *P. scaber* were isolated from the marsupium and fixed in 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2). Specimens were washed in cacodylate buffer and embedded in tissue freezing

medium (Jung). Transversal sections (10 μm) were cut with a Leica CM1850 cryostat at –18°C and stained with Alizarin red S solution in 0.2 M Trihydroxylmethyl aminomethane (Tris) - HCl buffer (pH 9). Cuticle of adult *P. scaber* was used as a positive control. Sections were imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRC Axiocam camera.

Results

Ontogenetic development of *Porcellio scaber* in the marsupium, from released fertilized eggs to marsupial mancas, was recently described morphologically (Wolff 2009, Milatovič et al. 2010), but the issue of protective envelopes was not addressed specifically. We present here the ultrastructural architecture of chorion, vitelline membrane and cuticle in embryos and marsupial mancas of isopod crustaceans *P. scaber* and *P. dilatatus*.

Ultrastructure of chorion and vitelline membrane

Both egg envelopes, distal chorion and proximal vitelline membrane, surround early-stage (Figs 1A, B, C, E) and mid-stage embryos (Figs 2A, B). Chorion has a similar appearance in both stages. It is a one-layered envelope, separated from the embryo surface and is approximately 500 nm thick. Ultrastructurally chorion consists of an electron dense matrix with sparse electron lucent "lacunae" (Figs 1D, 2C, 2D).

The vitelline membrane surrounds embryos throughout the whole developmental period (Figs 1A, 1 B, 1E, 2A, 2B, 3A, 3B, 3E, 4A). It maintains the same thickness of approximately 200 nm from early-stage till late-stage embryo. It is closely apposed to the embryo surface in early-stage embryos (Figs 1B, D, F), while it is slightly detached from the embryo surfaces of mid-stage embryos (Figs 2B, E, F) and late-stage embryos (Figs 3B, C, F, G). Above the embryo limb buds a wider space appears between embryo surface and vitelline membrane due to intense cell rearrangement during limb buds formation (Fig. 2B). The vitelline membrane consists of a thick proximal homogenous electron dense matrix superposed by a thin middle electron dense layer and a superficial corrugated lucent layer (Figs 1F, 2E, 2F, 3C). In non-osmicated specimens of late-stage embryo, the main thick layer is evidently lighter and superficial layer is not discerned (Fig. 3D). Between the outer embryo surface and the vitelline membrane of late-stage embryo a network of fibers was observed by SEM, presumably functioning as connective elements (Figs 3F, G).

Structure and renewal of cuticle

In late-stage embryo the embryo surface is covered with a homogenous extracellular matrix (Figs 3C, F, G). The prehatching late-stage embryo is the earliest developmental

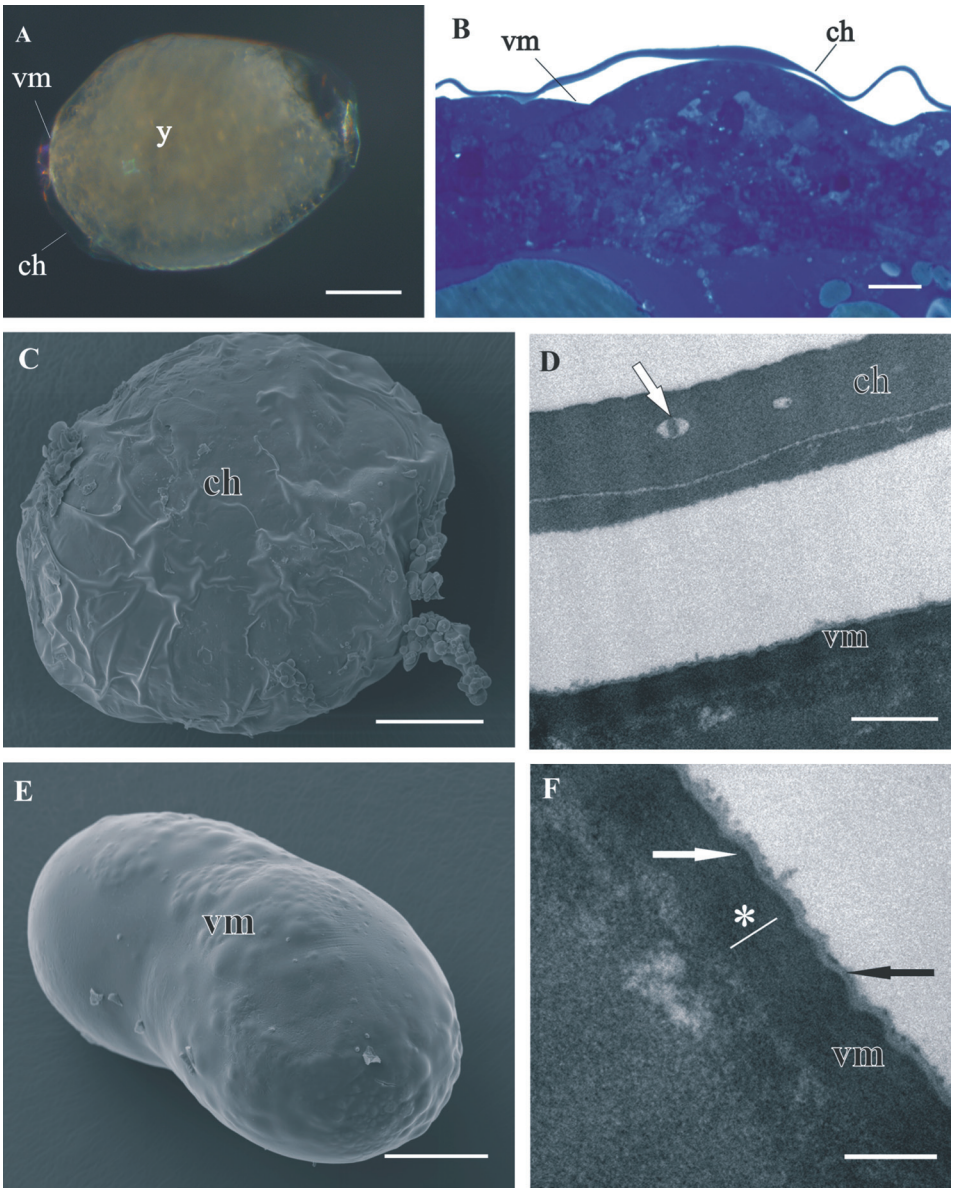

Figure 1. Structure of distal chorion (**ch**) and proximal vitelline membrane (**vm**), covering *P. scaber* **A, B, D, F** and *P. dilatatus* **C, E** early-stage embryo. **A** The early-stage embryo with large amount of yolk (**y**) and no visible limb buds. **B** Semithin section of the embryo peripheral region. Chorion is separated from the embryo surface. The vitelline membrane is closely apposed to the embryo surface. **C** SEM micrograph of the early-stage embryo. The outer egg envelope, chorion, is visible. **D** TEM micrograph of one-layered chorion, including electron lucent "lacunae" (white arrow). There is a layer of artificially spilt yolk underneath the chorion. **E** SEM micrograph of the early-stage embryo. Chorion is artificially removed and the inner egg envelope, vitelline membrane, is exposed. **F** TEM micrograph of vitelline membrane, composed of three layers: main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Bars: **A, C, E** 200 μm; **B** 10 μm; **D** 0.5 μm; **F** 200 nm.

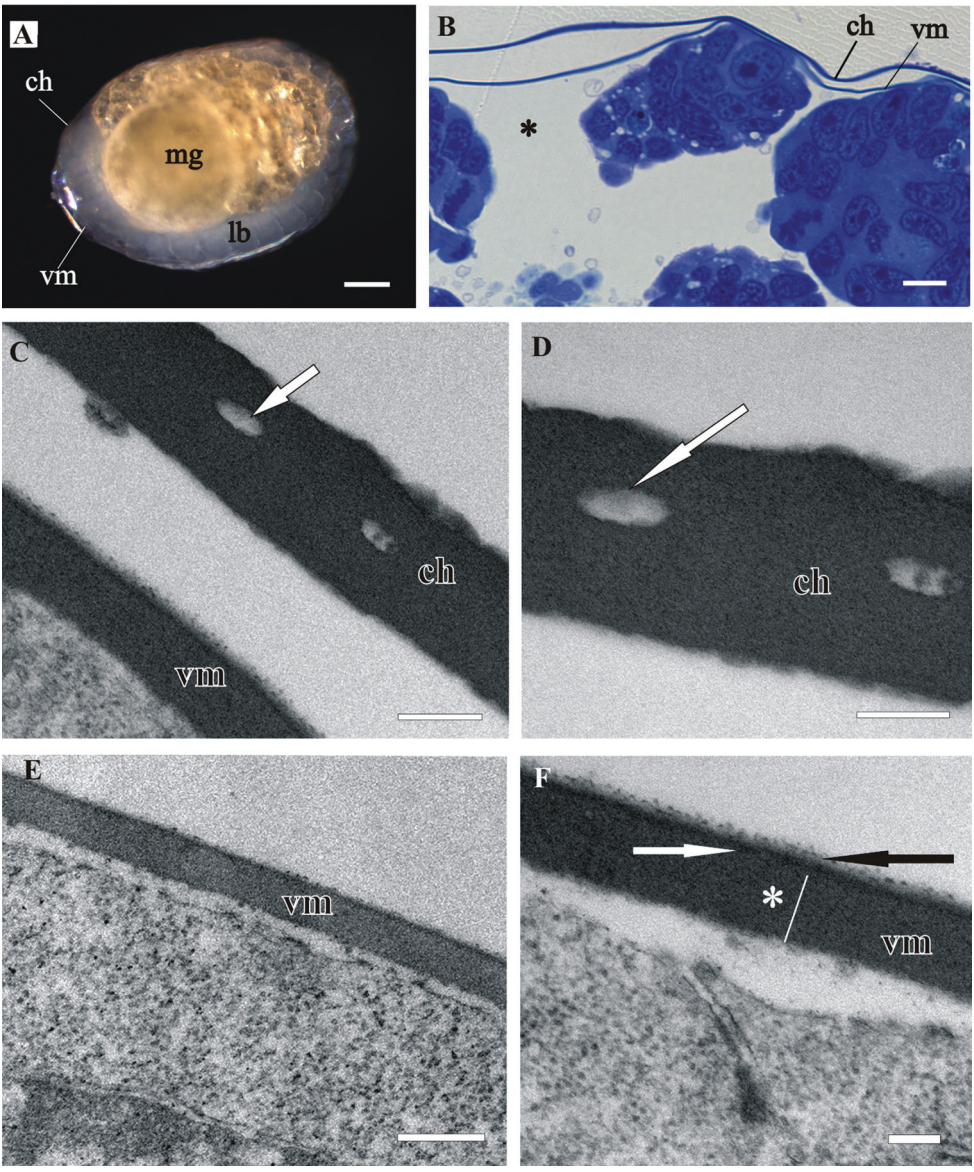

Figure 2. Structure of distal chorion (**ch**) and proximal vitelline membrane (**vm**), covering *P. scaber* mid-stage embryo. **A** The mid-stage embryo with visible limb buds (**lb**) and midgut glands primordia (**mg**). **B** Semithin section of the embryo peripheral region. Chorion is separated from the embryo surface. The vitelline membrane is slightly detached from the embryo cells; * - a wider space between embryo surface and vitelline membrane. **C, D** TEM micrographs of one-layered chorion, including electron lucent "lacunae" (white arrow). **E, F** TEM micrographs of vitelline membrane, composed of three layers: main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Bars: **A** 200 μm; **B** 10 μm; **C, E** 0.5 μm; **D, F** 200 nm.

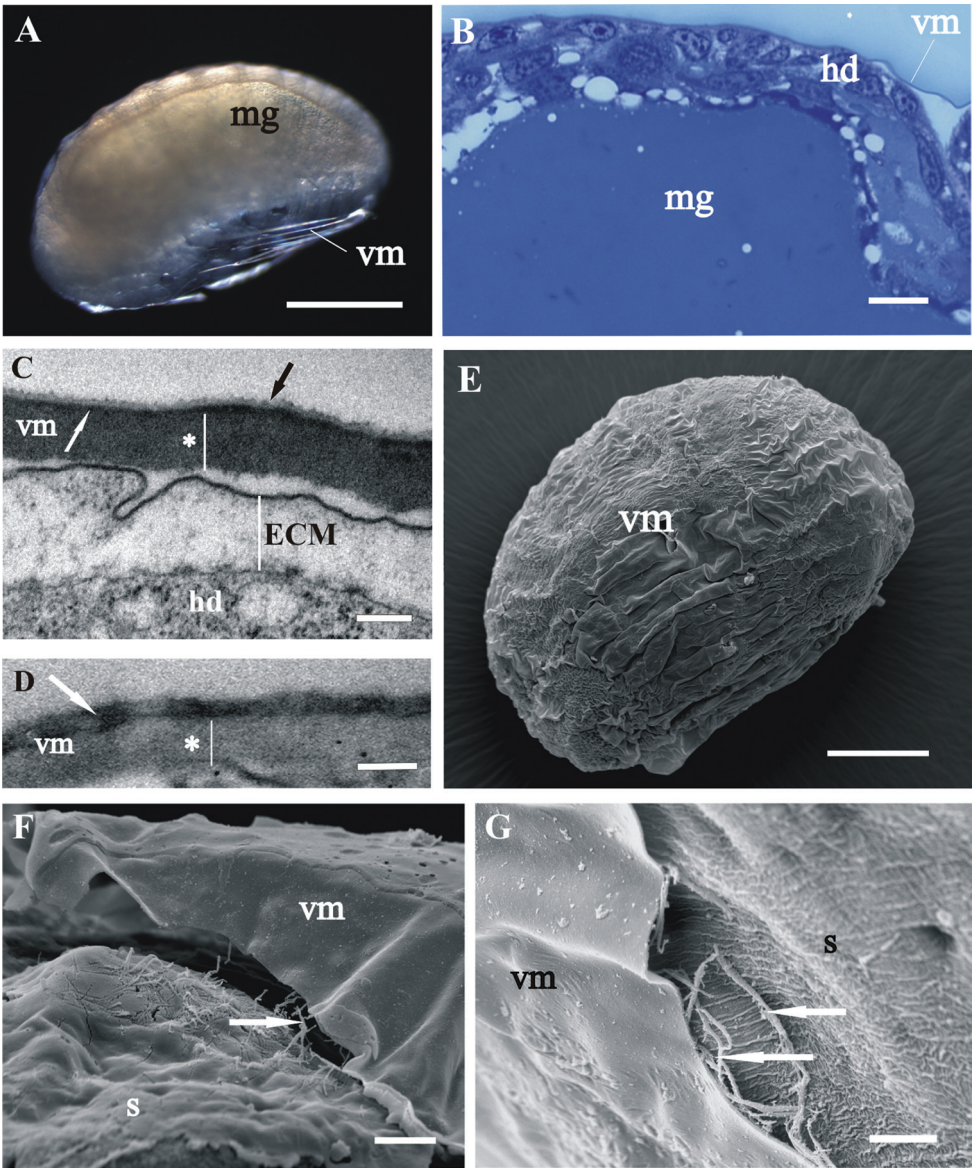

Figure 3. Structure of vitelline membrane (**vm**), covering *P. scaber* **A–D** and *P. dilatatus* **E–G** late-stage embryo. **A** Ventrally bent late-stage embryo, yolk is completely enclosed into the midgut glands (**mg**). **B** Semithin section of the embryo peripheral region. Vitelline membrane is slightly detached from the hypodermis (**hd**). **C, D** TEM micrographs of the vitelline membrane in osmicated specimen **C** and in non-osmicated specimen **D** Main proximal homogenous layer (*), thin middle electron dense layer (white arrow) and superficial corrugated lucent layer (black arrow). Hypodermis is covered with an extracellular matrix (ECM). **E** SEM micrograph of the late-stage embryo surrounded by vitelline membrane. **F, G** SEM micrographs of the late-stage embryo surface area. The vitelline membrane is artificially slit and fibers (arrows) between the outer embryo surface, covered with an extracellular matrix (s), and the vitelline membrane are exposed. Bars: **A** 500 μm; **B, F** 10 μm; **C, D** 200 nm; **E** 200 μm; **G** 5 μm.

stage, in which the overall ultrastructural architecture of the cuticle is similar to the adult crustacean cuticle and first morphological evidence of cuticle renewal is evident. Cuticle is from 2 to 3 μm thick, which is significantly thinner than in adults. It is composed of three layers, the outermost thin electron dense epicuticle, the middle exocuticle and the innermost endocuticle (Figs 4C, D, E). Cuticular scales are fully elaborated (Fig. 4B, D). Several transverse sections of completely structured sensilla are observed in the hypodermis (Fig. 4F). Dendritic outer segments and enveloping cells are clearly differentiated. The characteristic pattern of chitin-protein fibers arrangement in the exocuticle is resolved in some regions and hardly discernible in other regions of the same specimen. The endocuticle is subdivided in several electron dense sublayers alternating with electron lucent sublayers (Figs 4C, D, E). In some regions pore canals are visible running through the endocuticle, consisting of electron lucent central part and electron dense margins (Fig. 4C). Several morphological features in prehatching late-stage embryo show the exoskeletal cuticle renewal already in this stage of development: partly disintegrated proximal portion of endocuticle in some regions (Fig. 4C); cuticle detachment from the hypodermis (Figs 4B, C, D, E); rough apical plasma membranes of hypodermal cells and irregularly arranged electron dense particles on their outer surface (Figs 4C, E).

Next, we present evidence of cuticle renewal in different stages of marsupial mancas, namely: (I) early-stage marsupial manca, immediately after hatching (Fig. 5A); (II) mid-stage marsupial manca (Fig. 5B) and (III) late-stage marsupial manca, just prior to release from the marsupium (Fig. 5C). In the marsupial mancas the cuticle has similar architecture as the cuticle of prehatching embryo (Figs 5D–I). The main difference is the more pronounced characteristic chitin-protein pattern of exocuticle with a dense distal layer in marsupial mancas in comparison to embryo (Figs 5G, I). The micrograph of the late-stage marsupial manca cuticle displays well-formed pattern of exocuticle, resembling the adult helicoidal exocuticle structure and with a dense distal layer (Fig. 5I). The endocuticle of marsupial mancas is perforated by pore canals. The thickness of the marsupial manca cuticles is up to 3 μm. Morphological characteristics of cuticle renewal are observed in all three stages of marsupial mancas (Figs 5G-K). Apolysis, detachment of the old cuticle from the hypodermis is clearly visible in several stages (Figs 5F, H, I). The detached cuticle is partly degraded and much thinner in certain regions of the same specimen. The ecdysal space between the detached cuticle and the newly forming cuticle is also well evident and it contains homogenous electron dense material in some specimens (Fig. 5H), while in others it appears devoid of electron dense material (Fig. 5I). The newly assembling cuticle, covering hypodermal cells, consists of two layers, a thin electron dense external layer - epicuticle and an electron lucent procuticle (Figs 5H, I, K). In some regions of late-stage marsupial manca the procuticle appears homogenous (Fig. 5I), while in other regions helicoidal chitin-protein fibers arrangement is clearly discernible (Fig. 5K). The surface of the new cuticle is slightly (Fig. 5H) or highly wrinkled (Fig. 5I). Protrusions with electron dense tips are formed on apical surfaces of hypodermal cells. These characteristic features of cuticle

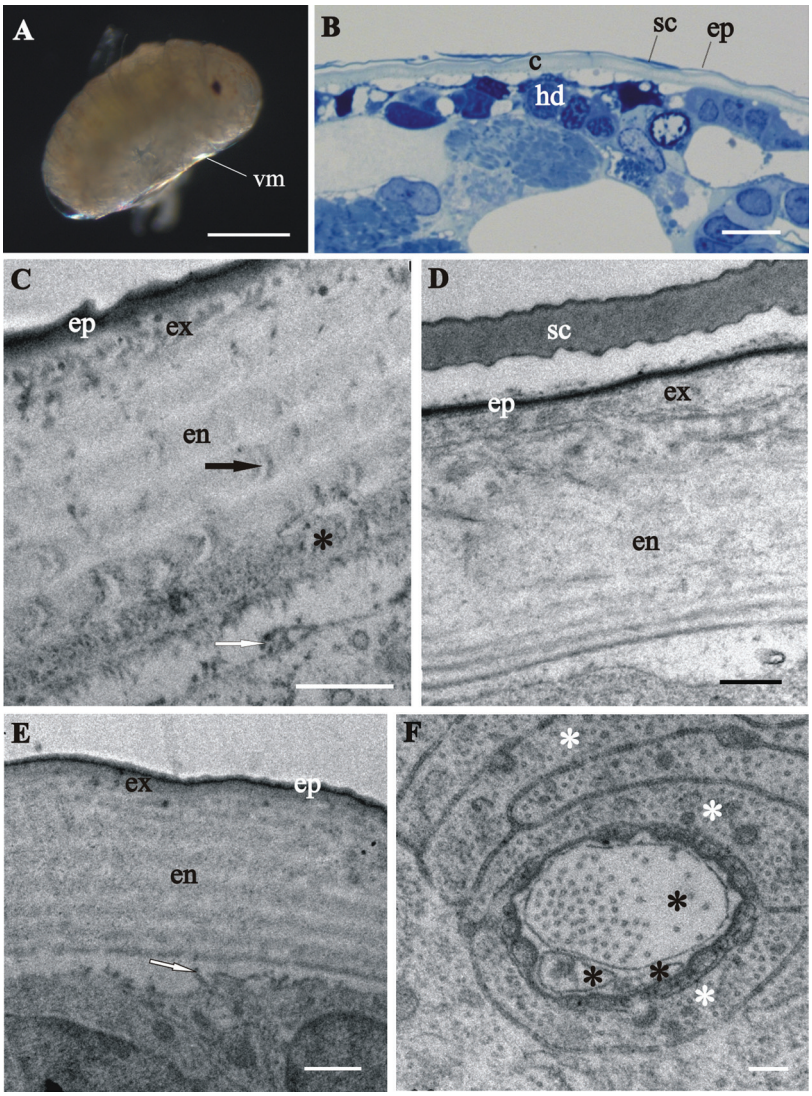

Figure 4. Cuticle structure and renewal in *P. scaber* prehatching late-stage embryo. **A** Swelled embryo inside the vitelline membrane (**vm**), prior to hatching. **B** Semithin section of the embryo peripheral region. The vitelline membrane is artificially removed. Clearly discernible exoskeletal cuticle (**c**), detached from the underlying hypodermis (**hd**). **C, D, E** TEM micrographs of exoskeletal cuticle in different regions of the same specimen, composed of three principal layers: the outermost thin electron dense epicuticle (**ep**), the middle exocuticle (**ex**) and the innermost endocuticle with several sublayers (**en**). The micrographs show features of cuticle renewal: cuticle detachment from the hypodermis, partial disintegration of proximal portion of endocuticle (*) and irregularly arranged electron dense particles on outer apical plasma membrane surface (white arrows). Pore canals (black arrow) in the endocuticle consist of electron lucent central part and electron dense margins **C**. Cuticular scales (**sc**) are fully elaborated and the exocuticle has the characteristic pattern of chitin-protein fibers arrangement **D**. Exocuticle is hardly discernible **E**. **F** TEM micrograph of completely structured sensillum transverse section in the hypodermis. Dendritic outer segments (*) and enveloping cells (white *). Bars: **A** 500 μm; **B** 10 μm; **C, E** 1 μm; **D** 0.5 μm; **F** 200 nm.

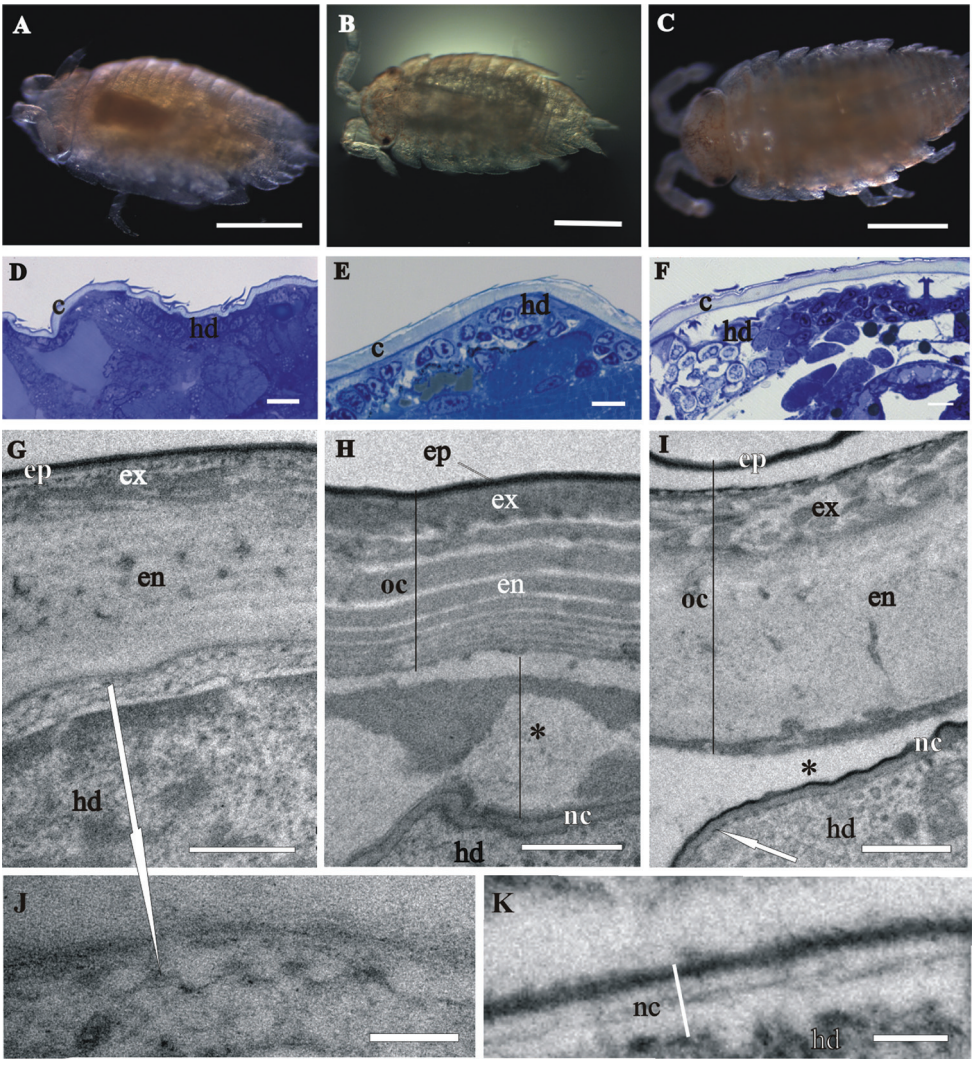

Figure 5. Cuticle structure and renewal in *P. scaber* marsupial mancas. **A** The early-stage marsupial manca, immediately after hatching **B** The mid-stage marsupial manca **C** The late-stage marsupial manca, just prior to release from the marsupium **D–F** Semithin sections of the manca peripheral region in the early-stage marsupial manca **D** in the mid-stage marsupial manca **E** and in the late-stage marsupial manca **F**. Cuticle (**c**), overlying the hypodermis (**hd**), becomes progressively more similar to adult cuticle. **G–K** TEM micrographs of exoskeletal cuticle in the early-stage marsupial manca **G, J** in the mid-stage marsupial manca **H** and in the late-stage marsupial manca **I, K** Three main layers are distinguished: epicuticle (**ep**), exocuticle (**ex**) and endocuticle (**en**). The micrographs show morphological characteristics of cuticle renewal: detachment of the old cuticle (**oc**) from the hypodermis, ecdysal space (*) between the detached cuticle and the newly forming cuticle (**nc**) and partial degradation of the old cuticle **H, I** protrusions with electron dense tips (white arrows) on apical surfaces of hypodermal cells **G, I, J**. The new cuticle consists of two layers, external electron dense epicuticle and internal electron lucent procuticle **H, I, K**. Helicoidal chitin-protein fibers arrangement is discernible in some regions of late-stage marsupial manca **K**. Bars: **A–C** 500 μm; **D–F** 10 μm; **G–I** 1 μm; **J, K** 200 nm.

synthesis are clearly visible underneath the assembling cuticle in all stages of marsupial mancas (Figs 5G, I, J).

The results of histochemical reaction with Alizarin red S for calcified tissues localization indicate that the cuticle of marsupial mancas is not strongly mineralized (Fig. 6A) in comparison to adult cuticle (Fig. 6B). The ventral calcium deposits, characteristic for adult premolt animals, were not observed in molting marsupial mancas.

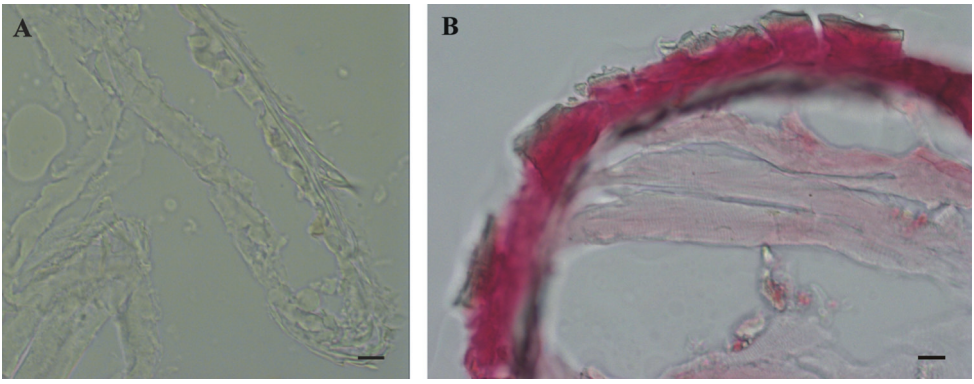

Figure 6. Histochemical reaction for calcium – Alizarin red S. **A** No reaction in *P. scaber* late marsupial manca **B** Positive control (red-pink) in adult *P. scaber* cuticle. Bars: 10 μm.

Discussion

The structure of egg envelopes and the ultrastructural architecture of the exoskeletal cuticle in *Porcellio* embryos and marsupial mancas share some common features with those described in other arthropods, but there are also some significant differences.

In comparison to egg envelopes of *Drosophila* eggs from ovaries, described by Margaritis et al. (1980), the egg envelopes of *Porcellio* embryos are thinner and have considerably less complex ultrastructure. Chorion of *Porcellio* is approximately two times thinner than chorion of *Drosophila.* The chorion of *Drosophila* eggs is differentiated into several layers, a thin innermost chorionic layer, a complex fenestrated endochorion and a fibrous exochorion, while *Porcellio* chorion in embryos consists of a single homogenous layer. Electron lucent "lacunae" observed in the *Porcellio* chorion resemble the cavities in the inner part of the *Drosophila* chorion. These complex cavities are thought to be air-filled in laid eggs and involved in respiration (Margaritis et al. 1980). Study of embryo tolerance to physiological stresses in terrestrial isopod *Armadillidium vulgare* shows that the chorion has low permeability to water and solutes and contributes to the very high tolerance to osmotic stress of early-stage embryo (Surbida and Wright 2001). The vitelline membrane of *Porcellio* embryos also appears thinner, but has a similar ultrastructure as *Drosophila* eggs vitelline membrane. Our results indicate that the osmiophilic main proximal layer and the thin superficial layer of *Porcellio* vitelline membrane differ in composition from the middle electron dense layer. The thin superficial layer may correspond to a similar thin layer above vitelline

membrane in *Drosophila* egg (Margaritis et al. 1980). The authors presume that this layer in *Drosophila* consists of wax, functioning to reduce water loss. This is not expected for *Porcellio* embryos since they are less vulnerable to desiccation due to their development in aqueous environment. The comparison of egg envelopes ultrastructure in *Porcellio* embryos and in *Drosophila* eggs reveals several differences. We consider these dissimilarities the consequence of different environments of embryonic development in *Porcellio* embryos which develop in a protected fluid-filled maternal brood pouch and in *Drosophila* embryos which are directly exposed to external environment during development. Here we also report on a network of fibers between embryo surface and vitelline membrane in late-stage embryo. We presume that these fibers function as connective elements between embryo and envelope, but no comparative data on vitelline membrane attachment were found in the literature.

We show here that the late embryo is already covered with a homogenous extracellular matrix, possibly first cuticle. In the prehatching late-stage embryo of *P. scaber* the exoskeletal cuticle has already main features of the adult crustacean cuticle, but minor differences are evident. It is composed of three principal layers - epicuticle, exocuticle and endocuticle. Endocuticle shows the typical arrangement of chitin lamellae in sublayers which are not so distinct as in adult cuticle. The characteristic pattern of chitin-protein fibers arrangement in the exocuticle of adults is not discernible in exocuticle of the prehatching late-stage embryo. A distal layer, similar to the dense distal exocuticular layer, described in adult isopods (Hild et al. 2009, Huber and Ziegler 2011, Seidl and Ziegler 2011), is not observed in the cuticle of prehatching embryo. Sensilla in hypodermis are already very elaborated and have similar ultrastructure as tricorn sensilla described in adult *P. scaber* (Ziegler and Altner 1995). Previous studies of cuticle differentiation during embryonic development of *Drosophila melanogaster* and *Parhyale hawaiensis* show that cuticle ultrastructure in last stage embryo likewise resembles adult cuticle ultrastructure, with typical helicoidal arrangement of chitin lamellae in procuticle and several-layered epicuticle (Locke 2001, Moussian et al. 2006, Havemann et al. 2008, Moussian 2010). Our observations of hypodermal cells in the prehatching late-stage embryo are in agreement with those described in *Drosophila*, as they are both flattened, with centrally placed nucleus, scarce organelles and connected by septate junctions (Moussian et al. 2006). During development of *P. scaber* marsupial mancas cuticle becomes progressively more similar to adult cuticle, particularly regarding the characteristic pattern of chitin-protein fibers in exo- and endocuticle, which is more and more explicit. In the distal portion of the exocuticle an electron dense layer is clearly resolved. It could correspond to the dense distal exocuticular layer, described in several adult isopods (Hild et al. 2009, Huber and Ziegler 2011, Seidl and Ziegler 2011). Several authors report on possibility of exoskeleton calcification in marsupial mancas. Inferred only by increased total calcium concentration of *Armadillidium vulgare* late-stage marsupial manca, Ouyang and Wright (2005) suggest that cuticle calcification starts in this stage. Surbida and Wright (2001) presume that wide osmotic tolerance of *A. vulgare* marsupial mancas is a consequence of calcification of their cuticle. Havemann et al. (2008) report on cuticle calcification after hatching of amphipod

Parhyale hawaiensis embryo, but it is not known which larval stage was observed. Our research, using histochemical approach to localize calcified tissue, indicates that the exoskeletal cuticle of *P. scaber* marsupial mancas is not strongly, if at all mineralized. It could be possible that amorphous calcium carbonate was dissolved during preparation, since it has relatively high solubility.

Next, the issue of cuticle renewal during intramarsupial development was addressed in this study. Partly disintegrated proximal endocuticle and cuticle detachment from the underlying hypodermis indicate that cuticle renewal takes place already in the prehatching embryonic stage. Prehatching embryo is thus the earliest stage of *P. scaber* development, where renewal of exoskeleton, i.e. initiation of molting, was observed so far. These results are in agreement with the previous observation of apolysis on appendage tips in late-stage embryo of *P. scaber* (Milatovič et al. 2010). Apolysis and cuticle disintegration were not observed in the studies of cuticle structure during embryonic development in insect *Drosophila melanogaster* and amphipod crustacean *Parhyale hawaiensis* (Locke 2001, Moussian et al. 2006, Havemann et al. 2008, Moussian 2010). We observed that the old cuticle is detached from the hypodermis and the new cuticle is produced in marsupial mancas of different stages, which is a sign of premolt. Several similarities and differences with respect to molting process in adult isopods are described. Similarities in apical protrusions of hypodermal cells during cuticle synthesis, and appearance of ecdysal space are very explicit. Regarding synthesis of newly assembling cuticle prior to ecdysis we show that preecdysal cuticle in mancas has mostly homogenous procuticle, with no distinct chitin-protein arrangement, although in certain regions a helicoidal arrangement of chitin-protein fibers is evident. In adult *P. scaber* it is reported that several exocuticular lamellae are deposited in premolt stage (Ziegler 1997). Advanced stages of new cuticle formation in marsupial mancas need to be investigated in further research. Molting in two phases is typical in adult terrestrial isopods, while this is still not confirmed for developing marsupial mancas. In adult isopods some other morphological changes accompany molting process, particularly concerning calcium dynamics. In adult premolt stage ecdysal space in-between the old and new cuticle contains calcium storage granules (Ziegler 1994, Štrus and Blejec 2001). In mancas no similar granules were observed in the ecdysal space. In adults appearance of calcium deposits on the first four anterior sternites is a clear indication of the premolt stage (Steel 1982, Zidar et al. 1998). In our study calcium deposits in molting marsupial stages were not observed, indicating differences in calcium dynamics compared to adults. There are no data on calcium dynamics during marsupial development in terrestrial isopods.

Ultrastructural research of isopod egg envelopes and cuticle structure during ontogenetic development in a fluid-filled marsupium is a valuable approach to get insight into their differentiation and function and contribute to comparative analysis of ontogenetic development in different arthropods. Evaluation of the data on the newly forming cuticle structure and composition obtained in sequential phases of the ontogenetic development is important to get insight into the mechanisms of mineralized biological matrix assembly.

Conclusions

During marsupial development of *Porcellio scaber* embryos and marsupial mancas in different stages are coated by different protective envelopes (Fig. 7).

Egg envelopes of isopod crustacean *Porcellio scaber* embryos are thinner and structurally less complex in comparison to egg envelopes of insect *Drosophila melanogaster.* These are expected differences due to different embryonic developmental strategies of these arthropods. Similarities in egg envelopes of these two species appear particularly in their inner egg envelope, the vitelline membrane.

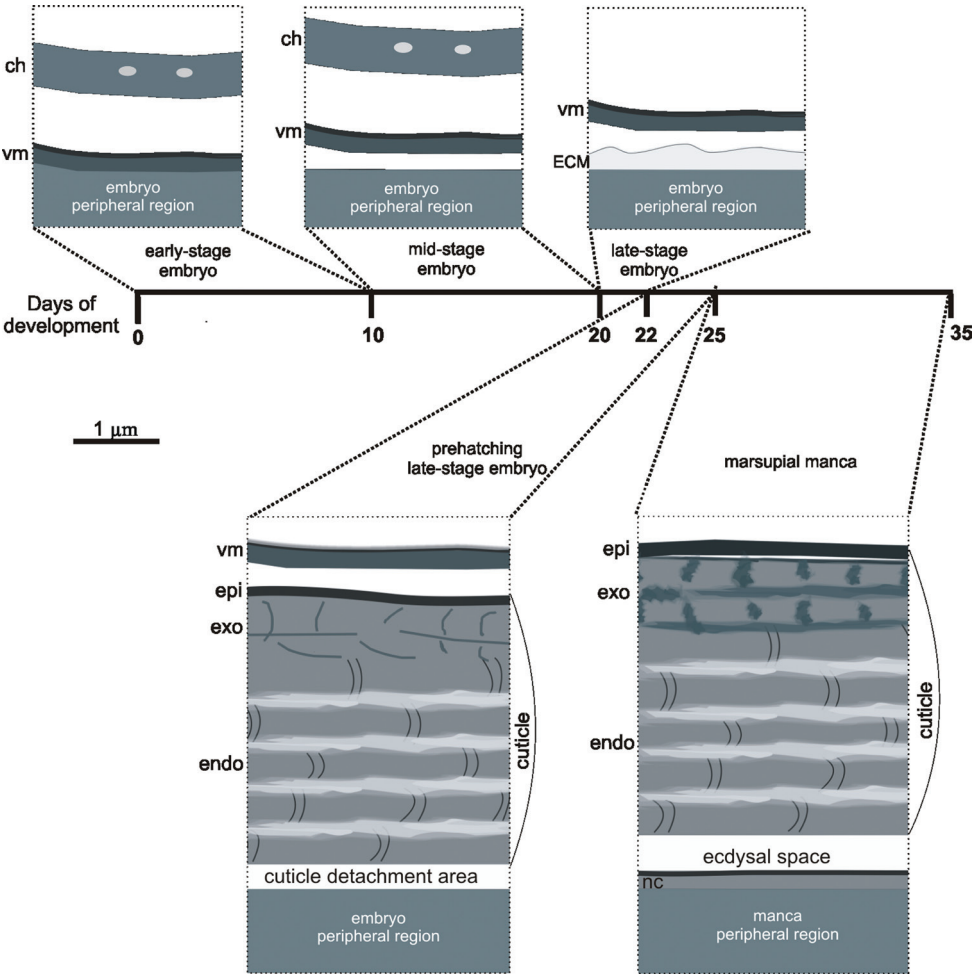

Figure 7. Schematic representation of different protective envelopes, coating *Porcellio scaber* embryos and marsupial mancas during development (lasting 35 days), namely egg envelopes (chorion and vitelline membrane) and exoskeletal cuticle. During growth of embryos and mancas egg envelopes are shed and cuticle is renewed. **ch** – chorion; **vm** – vitelline membrane; **ECM** – extracellular matrix; **epi** – epicuticle; **exo** – exocuticle; **endo** – endocuticle; **nc** – newly assembling cuticle.

Exoskeletal cuticles of *Porcellio scaber* prehatching late-stage embryo and marsupial mancas have already some features of the adult crustacean cuticle, but are significantly thinner. Three principal layers are distinguished, the outermost epicuticle, the middle exocuticle and the innermost endocuticle. Characteristic chitin-protein patterns of adult cuticle, particularly regarding the exocuticle, are not very distinct in prehatching late-stage embryo and become progressively more explicit in marsupial mancas. Cuticular scales and sensilla are fully elaborated already in prehatching late-stage embryo. In the distal portion of the exocuticle a dense layer is observed in marsupial mancas, which could correspond to the dense distal exocuticular layer of adult cuticle. Marsupial manca cuticle is not strongly calcified.

Cuticle renewal takes place already in prehatching late-stage embryo, where detachment of cuticle from hypodermis and partial disintegration of proximal endocuticle occur. Old cuticle detachment and new cuticle assembly appear in marsupial mancas of several stages. Morphological changes, related to calcium storage during molt cycle in adult isopods, were not observed in premolt marsupial stages.

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RESEARCH ARTICLE

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Electron microscopic and preparative methods for the analysis of isopod cuticle

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Abstract

The crustacean cuticle consists of a complex organic matrix and a mineral phase. The physical and chemical properties of the cuticle are corellated to the specific functions of cuticular elements, leading to a large variety in its structure and composition. Investigation of the structure-function relationship in crustacean cuticle requires sophisticated methodological tools for the analysis of different aspects of the cuticular architecture. In the present paper we report improved preparation methods that, in combination with various electron microscopic techniques, have led to new insights of cuticle structure and composition in the tergite cuticle of *Porcellio scaber*. We used thin sections of non-decalcified tergites and decalcified resin embedded material for transmission electron microscopy and scanning transmission electron microscopy. Etched sagittal planes of bulk tergite samples were analysed with field emission scanning electron microscopy. We have found a distinct distal region within the exocuticle that differs from the subjacent proximal exocuticle in the arrangement of fibres. Within this distal exocuticle chitin-protein fibrils assemble to fibres with diameters between 15 and 50 nm that are embedded in a mineral matrix. In the proximal exocuticle and the endocuticle fibrils do not assemble to fibres and are surrounded by mineral individually. Furthermore, we show that the pore canals are filled with mineral, and demonstrate that mild etching of polished sagittal cuticle surfaces reveals regions containing mineral of diverse solubility.

Keywords

Isopoda, cuticle, ultrastructure, *Porcellio scaber*

Introduction

The structural organisation of the crustacean cuticle has recently led to increasing interest because of its high structural and chemical variability. Furthermore, the outstanding me-

chanical properties of the crustacean cuticle have attracted attention to its high potential for biomimetic technical applications. The organic matrix of the arthropod cuticle is hierarchically organised (Vincent 2002, Nikolov et al. 2011). Chitin chains form crystalline alpha chitin fibrils (Carlström 1957) that are surrounded by protein (Blackwell and Weih 1980). These chitin-protein fibrils assemble to larger fibres that form planes in which all fibres are orientated in the same direction. Recent studies have shown, that in some species these planes are formed by fibrils that are not assembled to fibres (Nicolov et al. 2011, Seidl et al. 2011). Many of these planes are stacked upon another in a way that the direction of the fibrils/fibres is twisted by a small angle against that of the preceding plane, thereby forming a twisted plywood structure (Bouligand 1972). In slightly oblique transversal sections through the cuticle, the twisted plywood structure results in a virtual stratification marked by chitin-protein fibrils/fibres that are oriented parallel to the cutting plane (Giraud-Guille 1984). The turn of fibrils/fibres by 180 degrees defines one stack of planes. The distance between two planes with parallel fibrils/fibres is called stacking height. These stacks form three of the four principal layers of the cuticle: the exocuticle, the endocuticle and the innermost membranous layer. The epicuticle is the outermost principal layer and contains no chitin-protein fibrils or fibres but proteins and waxy layers that serve as a barrier for water loss in terrestrial arthropods (Compère 1991). In crustaceans the exocuticle and endocuticle are mineralised by calcite, amorphous calcium carbonate (ACC), and to some degree by amorphous calcium phosphate (Dillaman et al. 2005, Boßelmann et al. 2007, Neues et al. 2007, Hild et al. 2008, Neues et al. 2011).

The crustacean cuticle is an exoskeleton that provides support, protection against predation and environmental strains, sites for muscle attachment, and structures involved in receiving sensory information. The cuticle surrounds the whole animal and is subdivided into skeletal elements such as segments of the main body and legs, claws, mouthparts, eye lenses, etc. Flexible cuticular membranes often connect skeletal elements to allow for relative movements. The structure and composition of the cuticle are adapted to the function of a specific skeletal element and to the habitat of the animal (Neues et al. 2007, Hild et al. 2009). This results in a large variety of cuticle structure and composition requiring a comparative approach to understand the structure-function-composition relationship of crustacean cuticle.

In the present paper we describe improved preparation methods for the cuticle of terrestrial isopods using the tergite cuticle of *Porcellio scaber* as an experimental model. The cuticle of *P. scaber* and other related species has been studied previously (Price and Holdich 1980, Compère 1991, Štrus and Compère 1996, Štrus and Blejec 2001, Hild et al. 2008, 2009, Huber and Ziegler 2011, Mrak et al. 2011, Ruangchai et al. 2011, Seidl et al. 2011). Our results confirm most structural features found earlier and have led to new insights in the ultrastructure and composition of the tergite cuticle.

Methods

Porcellio scaber Latreille, 1804 were collected from biotopes near Ulm, Germany, kept in plastic containers filled with soil and bark, and fed with fresh potatoes and dry oak leaves. Animals in the intermoult stage were identified by the lack of external signs of moulting such as sternal $CaCO_3$ deposits. The intermoult stage was further verified during tergite sample preparation and structural analysis. Specimens with any signs of apolysis and samples with soft or incompletely developed cuticle were not used. We used the tergites of pereonites 2–7.

For transmission and low voltage scanning transmission electron microscopy (TEM, STEM) of the organic phase, in decalcified EPON resin embedded samples, dissection of the cuticle was performed in a decalcification/fixation solution containing 0.05 mol L^{-1} EDTA for decalcification, 2.5% glutaraldehyde and 2% paraformaldehyde for fixation of organic material, and 0.25 mol L^{-1} HEPES (pH 7.8) for buffering pH during decalcification. The samples were incubated in fresh solution for 34 days at 4 °C. During simultaneous decalcification and fixation, parts of the organic matrix, which emerge slowly from surrounding mineral and are thus exposed to the solution, are immediately fixed by the aldehyde. The decalcified tergites were then washed 3 times in bi-distilled H_2O for 10 minutes each and postfixed for 1 h in a solution containing 1% OsO_4 and 0.8% $K_4[Fe(CN)_6]$ (White et al. 1979). After postfixing, they were washed again 3 times in bi-distilled H_2O for 10 minutes each and dehydrated in a graded series of isopropanol. Part of the samples was block contrasted in a solution of 0.5% uranyl acetate in denatured ethanol for 30 minutes, followed by 3 washing steps in ethanol for 4 minutes each. Then the samples were washed two times in acetone for approximately 4 minutes each and embedded in EPON resin. Sagittal, 50 nm thick sections of the embedded tergites were cut using an Ultracut ultramicrotome (Leica). The sections were placed on 300 mesh copper grids and analysed using a Jeol 1400 TEM at 80 kV acceleration voltage or with a Hitachi S-5200 field emission SEM (FE-SEM) operated at 30 kV.

STEM is a method where the electron beam is focused in a spot which is raster scanned across a sample. We used a high-resolution FE-SEM equipped with a dark field STEM-detector to perform STEM analysis at low voltages (30 kV), which considerably enhances contrast and still yields spatial resolution of about 1 nm. The contrast arises from local density of the sample that is in particular useful for the analysis of unstained sections of non-demineralised material.

For microscopy of non-decalcified samples, dissection in aqueous solutions should be avoided because this would lead to dissolution of the mineral phase within the endocuticle. *In vitro* experiments have demonstrated that the ACC polymorph is ten times more soluble than its crystalline counterparts (Brečević and Nielsen 1989), and it has been shown that the endocuticle of isopod cuticle contains ACC as the main mineral component. Although ACC is stabilised in biological tissues, preliminary experiments have shown that it quickly dissolves in aqueous solutions even at strongly basic pH values. Therefore, we used 100% methanol for dissection, in which ACC remains stable for at least one month (Becker et al. 2003). After dissection samples were washed

in bi-distilled water for 1–2 seconds to remove tissue saline at the surface and then for 25 seconds in 100% methanol to remove water. The specimens were left to air dry at room temperature (Hild et al. 2009).

To obtain polished sagittal faces and sections of native (non-demineralised) cuticle, tergites were first glued to cylindrical aluminium holders (diameter 3 mm) using super glue gel. Sagittal planes were cut with a glass knife and then polished with a Diatome diamond knife according to the method described previously (Fabritius et al. 2005). A Diatome ultra 35° diamond knife and a Diatome static line II ionisator were used to cut dry 60 nm thick sections. The sections were transferred to carbon coated formvar film on 300 mesh copper grids using an eyelash. To flatten the sections, another carbon coated formvar film on a 300 mesh copper grid was placed onto the grid carrying the sections. Sections were flattened by the weight of a brass-rod 3 mm in diameter and approximately 200 mm long with a polished end-surface. Finally, sections were coated with a 4–8 nm thick layer of carbon using a BAF 300 freeze-etch device (Balzers) and analysed by low voltage STEM (Hitachi S-5200 at 30 kV).

To expose the organic matrix in non-decalcified tergite cuticle, samples were first polished as described above and then etched for 20 seconds with an aqueous solution of 2.5% glutaraldehyde and 0.01 mol L^{-1} MOPS buffer adjusted to pH 6.5 or 0.1 mol L^{-1} HEPES buffer adjusted to 8.0. Then, the samples were washed 3 times for 10 minutes with isopropanol, critical point dried, rotary shadowed with 4 nm of platinum (BAF 300, Balzers) and analysed with a Hitachi S-5200 FE-SEM at 4 kV using the secondary electron detector. Secondary electrons (SE) have a low energy and thus only those elicited from near the sample surface can reach the detector and can thus contribute to imaging of the surface topography. A SEM with a field emission gun was used to obtain high resolution (nominally 1.8 to 0.5 nm at 1 to 30 kV, respectively) at low acceleration voltages as a result of the small diameter of the focused electron beam (spot size).

Results

Decalcified thin sections

TEM and STEM analyses of simultaneously decalcified and fixed samples provide detailed information about the specific hierarchical organisation of the organic matrix of the cuticle from its principal layers down to single protein fibrils and chitin crystallites. Exo- and endocuticle can be clearly distinguished by differences in contrast and morphology (Figure 1). The organic matrix of the exocuticle is more intensely stained than the endocuticle one, resulting in a sharp border between them (Figure 1A). In the proximal exocuticle pore canals are more branched than in the endocuticle and a high variation in the width of pore canals can be observed, probably due to twisting of spindle-shaped pore canals. In sagittal sections, this leads to an islelike appearance of the part of the organic matrix that is organised in a twisted plywood structure (Figures 1A, B). In the sagittal sections of the endocuticle however, pore canals have a lunulate ap-

pearance. The width of the pore canals decreases towards proximal stacks (Figure 1A). There is a clear difference in the organisation at the level of fibrils and fibres between a distinct distal and the proximal region of the exocuticle. Within the distal region of the exocuticle chitin-protein fibrils form fibres consisting of electronlucent approximately 3 nm thick chitin crystallites surrounded by densely stained protein (Figures 1C, D). The fibres vary in size and shape. Most fibres have a diameter of approximately 25 nm but fibres with diameters up to 50 nm also appear regularly. Within the proximal part

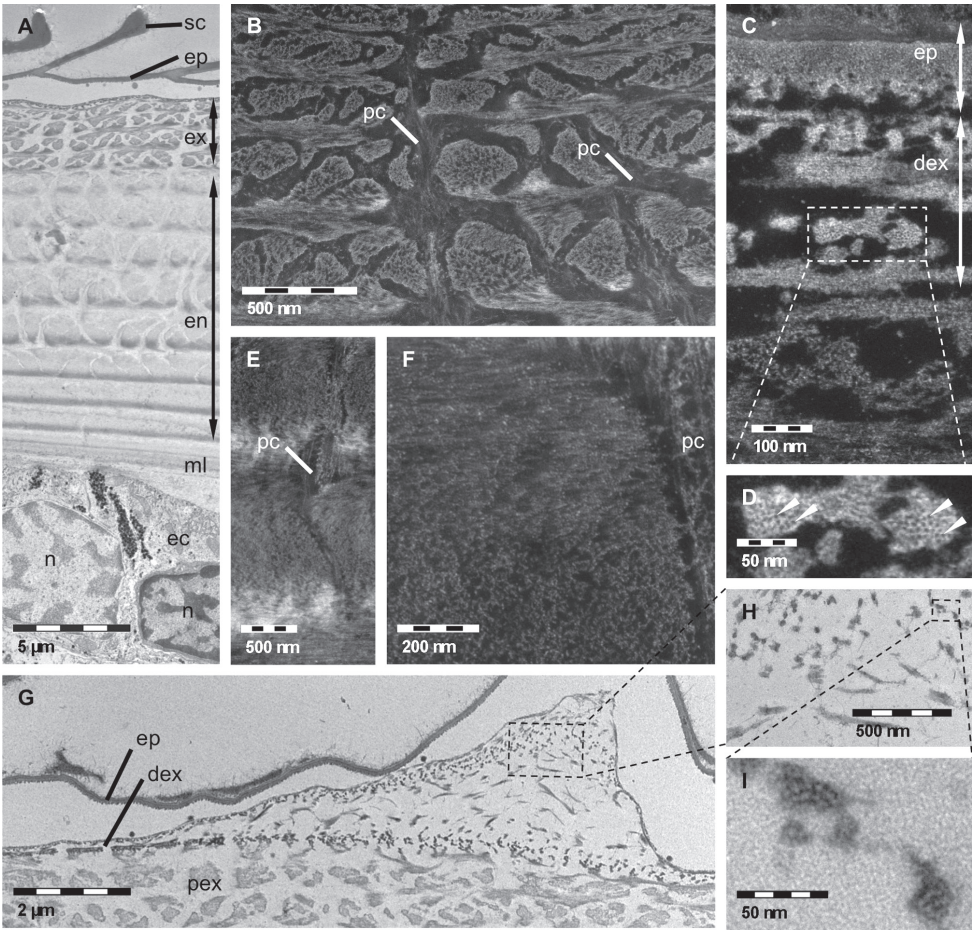

Figure 1. TEM **A, G, H, I** and STEM **B, C, D, E, F** micrographs of decalcified and EPON embedded tergites of *Porcellio scaber*. **A** Sagittal overview showing epicuticle (ep), exocuticle (ex), endocuticle (en) and membranous layer (ml), ec, epithelial cell; n, nucleus; sc, epicuticular scale. **B** Proximal exocuticle. Dense network of pore canals (pc) containing fibrils or fibres following the direction of the pore canal. **C, D** Fibres of the distal exocuticle (dex) consisting of approximately 3 nm thick unstained chitin crystallites (arrowheads) surrounded by densely stained proteins. **E, F** In the endocuticle single fibrils form the twisted plywood structure. The pore canals contain vertical fibrils or fibres. **G, H, I** Section through a cuticular thickening. The increase in cuticle thickness is brought about by an increase of the stacking height in the distal exocuticle only. **H, I** Details of **G** confirm the typical structure of fibres within the distal exocuticle.

of the exocuticle as well as in the endocuticle, fibrils appear not to form fibres (Figures 1E, F). Local thickenings of the cuticle, regularly observed in thin sections and probably representing edges of tubercles, derive from an increase in the height of stacks in the distal exocuticle, whereas the thickness of stacks of the endocuticle remains unchanged (Figures 1G–I). The 150 nm thick epicuticle consisting of an electron-lucent outer and an electron-dense inner epicuticle (Figure 1C) remains unaltered as well (Figure 1G).

Non-decalcified thin sections

Thin sections of non-decalcified cuticle are of value for studying the distribution of mineral and its spatial relation to the organic phase. Figure 2A shows a sagittal section through the whole cuticle. We confirm that the membranous layer and most regions of the outer epicuticle are devoid of mineral (Figures 2A, C). It appears that parts of the inner epicuticle and perhaps epicuticular pore canals may well contain mineral (Figures 2B, C). It is of interest that within the whole exo- and endocuticle pore canals are filled with mineral (Figures 2A, B, D). Thin pore canals within proximal stacks of the endocuticle can well be seen (Figure 2A). In the 11.5 μm thick distal exocuticle mineral surrounds electron-lucent organic fibres approximately 25 nm in diameter indicating that the proteinaceous material around the chitin crystallites shown in Figures 1C and 1D remains unmineralised (Figure 2B). Within the proximal exocuticle and the whole endocuticle, we observe electron-lucent structures with diameters of approximately 6 nm, corresponding to single chitin-protein fibrils (Figures 2D, E). They are surrounded by mineral that forms interconnected tube-like structures, confirming that in these layers the fibrils do not assemble to fibres. At the border between the endocuticle and the membranous layer rod-like mineral structures occur in a partly mineralised region (Figures 2F, G).

Etched surface samples

Etched surfaces are in particular suitable to visualize the distribution of organic fibrils or fibres within the pore canal system. In addition, because shrinkage due to demineralisation followed by drying of the cuticle can be minimised, some quantitative aspects of the cuticle, such as the stacking height and thickness of exo- and endocuticle can be measured more accurately. Just as in non-decalcified thin sections the distal exocuticle is approximately 1 μm thick. Fibres within the pore canals that are only faintly visible in TEM sections can be well observed (Figure 3A). Etching of polished samples can also be used to distinguish mineralised from non-mineralised structures. A side view of a tricorn sensillum is shown in Figure 3B. Non-mineralised epicuticular structures in the cutting plane can well be distinguished from surrounding mineralised cuticular layers (Figures 3B, C). Depending on the pH used for etching polished and etched cuticle surfaces can be used to discern regions differing in the solubility of the mineral phases e.g. between ACC and calcite. Etched at pH 6.5, fibres or fibrils in all principal layers are visible

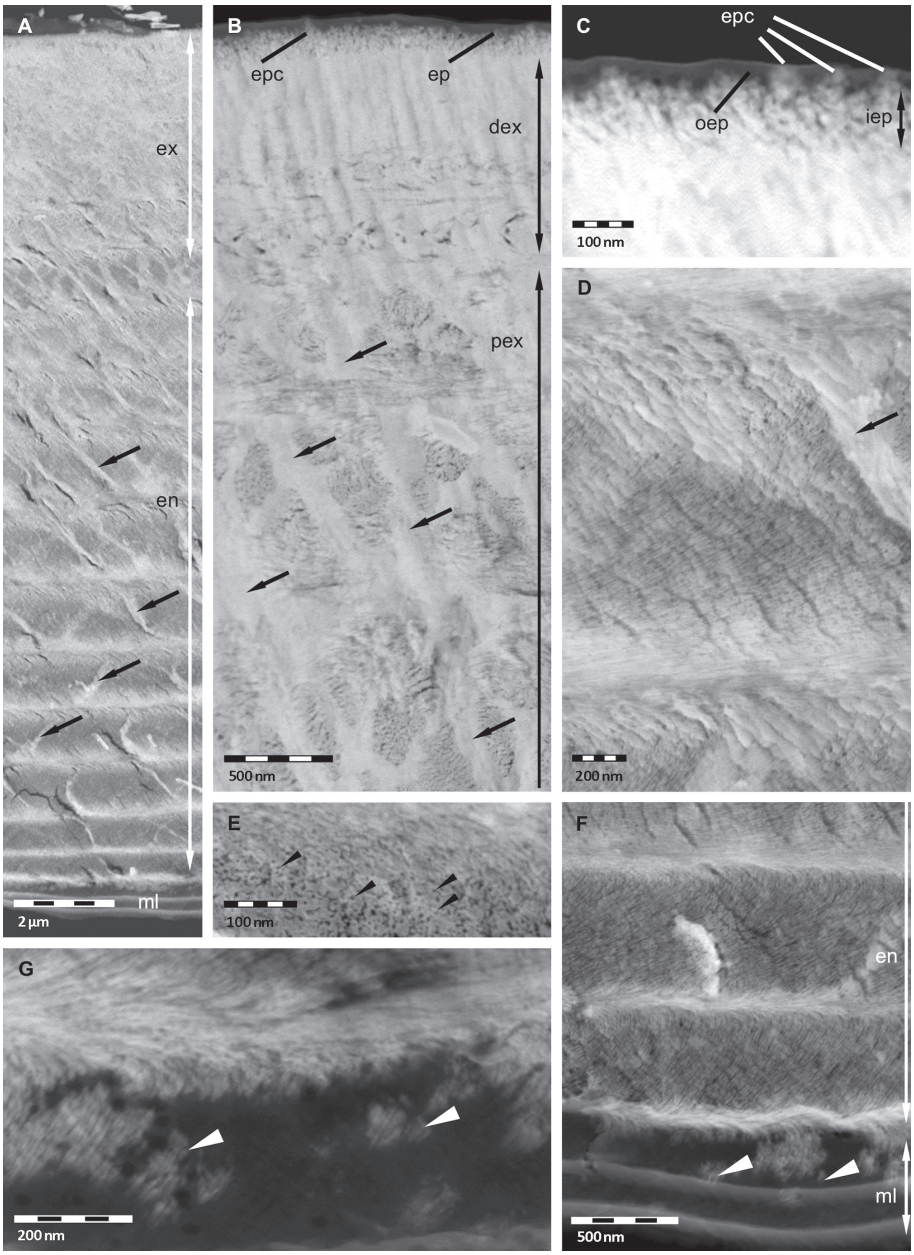

Figure 2. STEM micrographs of non-decalcified tergites of *Porcellio scaber*. **A** Overview showing the mineralised exocuticle (ex) and endocuticle en and the unmineralised membranous layer (ml). The pore canals (arrows) are mineralised. **B, C** Exocuticle and epicuticle (ep). Approximately 25 nm thick fibres in the distal exocuticle (dex) and approximately 6 nm thick fibrils in the proximal exocuticle (pex). Pore canals arrows contain mineral. The inner epicuticle (iep) appears partly mineralised, and the outer epicuticle (oep) unmineralised, except epicuticular pore canals (epc). **D, E** Endocuticle with mineralised pore canal (arrow). Approximately 6 nm thick chitin-protein fibrils (black arrowheads) individually surrounded by mineral forming a twisted plywood structure. **F, G** Single mineral rods (white arrowheads) at the border between membranous layer and endocuticle.

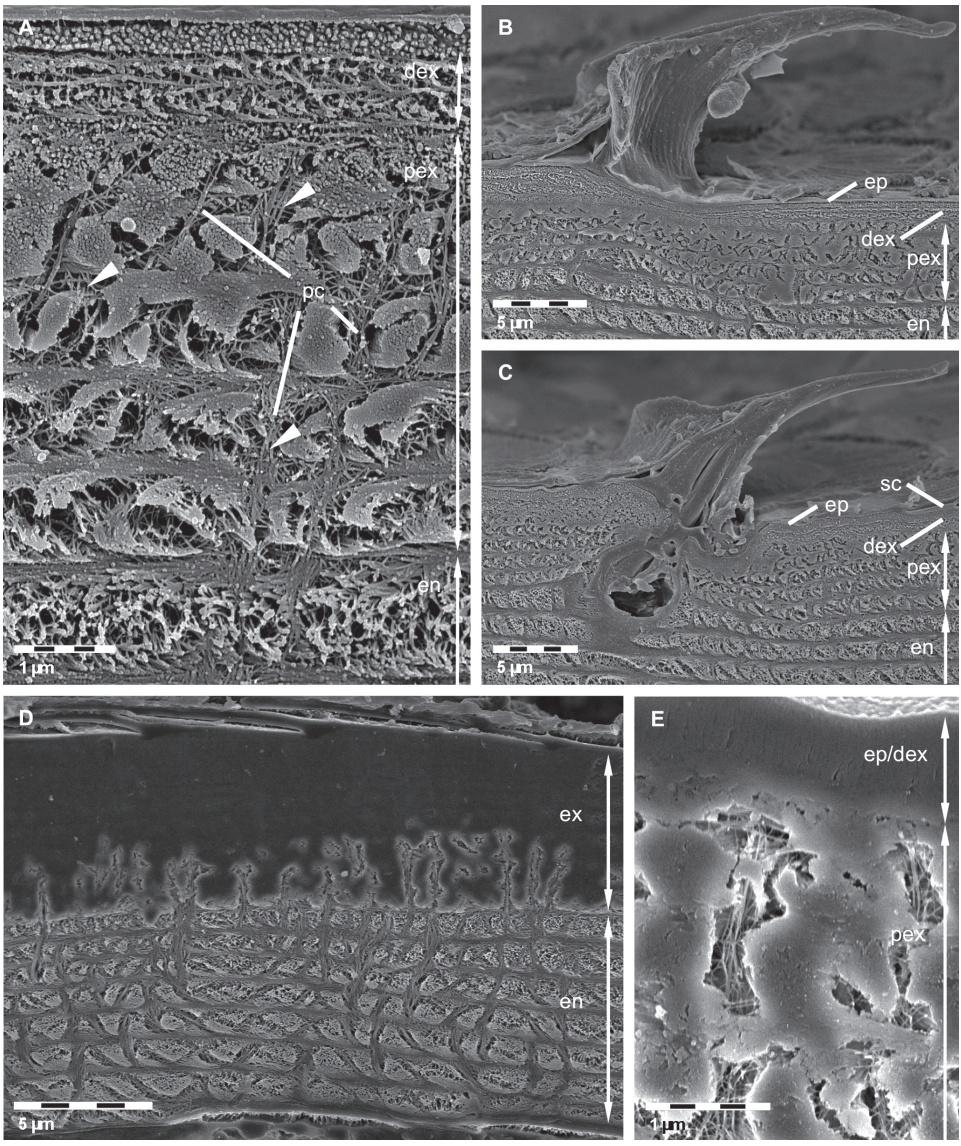

Figure 3. FE-SEM micrographs of polished sagittal plane through bulk tergite samples etched at pH 6.5 **A, B, C** and 8.0 **D, E**. **A** Fibres in the distal exocuticle (dex) and the isle-like structure of the proximal exocuticle (pex) caused by large pore canals (pc). Fibrils or fibres (arrowheads) in the pore canals are well visible. en, endocuticle. **B, C** Side views of tricorn sensilla. The epicuticular unmineralised material forming the sensilla is well distinguishable from the mineralised exo- and endocuticle. ep, epicuticle; sc, epicuticular scale. **D, E** Mild etching reveals regions containing mineral of different solubility. Mineral within the endocuticle appears etched whereas most regions within the exocuticle (ex) remain unaltered. Note etching within pore canals of the exocuticle.

(Figures 3A–C). At pH 8.0, mineral was etched away in the endocuticle and partly in the exocuticle (Figure 3D). In the latter etching occurs mostly in proximal regions of the pore canals, sometimes also more distally, but not in the distal exocuticle (Figures 3D, E).

Discussion

Using improved preparation methods for conventional TEM, low voltage STEM and FESEM, we can confirm all general features of the tergite cuticle described in several previous publications on the integument cuticle of terrestrial isopods (Price and Holdich 1980, Štrus and Compère 1996, Ziegler 1997, Štrus and Blejec 2001). In addition, we describe features that have not been described previously in the cuticle of *P. scaber*, such as the presence of a distinct distal exocuticle that differs from the subjacent proximal exocuticle, pore canals that contain mineral, the spatial arrangement of mineral around fibres within the distal exocuticle versus mineral around fibrils in the proximal exocuticle and endocuticle, and the distribution of well soluble mineral phases.

Simultaneous decalcification and fixing of samples allows for visualising the structure on fibre level within the distal exocuticle such as chitin crystallites embedded in a proteinaceous matrix. In *P. scaber* these fibres have the same structure as those reported previously for the distal exocuticle in the tergites of *Tylos europaeus* (Seidl et al. 2011). Interestingly, the relative size of distal and proximal exocuticle differs between these species. In *T. europaeus*, the distal exocuticle is between 4 and 10 µm thick and consists of at least 4 stacks versus 11.5 µm thick and just 12 stacks in *P. scaber.* In reverse, the proximal exocuticle consists of 2 stacks in *T. europaeus* and 4 stacks in *P. scaber*. The distal exocuticle appears to play a specific role in the formation of local thickenings in the tergites of *P. scaber* (present study) and the formation of micro-tubercles in *T. europaeus* (Seidl et al. 2011), in which thickening of the cuticle occurs by an increase in the height of the stacks in the distal exocuticle.

Studies on the distribution of calcite and ACC within the cuticle of three terrestrial isopods using Raman spectroscopy and Raman imaging techniques (Hild et al. 2008, 2009) have shown that calcite occurs within the exocuticle with little ACC in the proximal region, whereas the endocuticle contains ACC only. Because of the high solubility of ACC (Brecevic and Nielsen 1989) versus calcite at pH 8.0, the difference in etching of the surfaces on polished exo- and endocuticle confirms that only the exocuticle contains calcite. In regions of the exocuticle containing well soluble mineral phases such as ACC, etching exposes pore canals that can be identified by their vertically oriented fibrils or fibres. This suggests that ACC within the exocuticle is mostly localized within pore canals.

Previous structural analysis of broken non-demineralised cuticle revealed two regions in the exocuticle of *P. scaber*, *Armadillidium vulgare*, *Titanethes albus* und *T. europaeus*: a distal region in which broken faces appear smooth, hardly exposing any organic fibres (smooth layer), and a subjacent exocuticular layer in which stacks of mineralised twisted fibrils are well visible (Hild et al. 2008, 2009, Seidl et al. 2011). It is of particular interest that, according to the present results and those on the Tylidae *T. europaeus*, the thickness of the distal exocuticle appears to correlate with the smooth layer, although in this species peculiar polyhedral textures occur in addition to the smooth regions. Furthermore, we recently confirmed this correlation for the Tylidae *Helleria brevicornis* (Seidl and Ziegler 2011). Together, these observations suggest that

the organisation of the organic matrix at the level of fibres and fibrils may influence the fracture behaviour of exocuticular layers. This is in accordance with recent results on multi-scale modelling of crustacean cuticle that stress the importance of structural properties on the mechanical behaviour at all hierarchical levels including those at the microscale (Nikolov et al. 2010, 2011, Fabritius et al. 2011).

According to the model for insect cuticle, proteins form a helicoidal sheath around 19 anti-parallel chitin chains that form the approximately 3 nm thick crystallites (Blackwell and Weih 1980, Neville 1998, Vincent and Wegst 2004). The thickness of organic fibrils within non-decalcified thin sections of the endocuticle and proximal exocuticle presented here is in good agreement with the size of chitin-protein fibrils in the insect model. Thus it appears that in these layers single fibrils are surrounded by mineral and do not assemble to fibres, in contrast to the organic matrix of the distal exocuticle in which bundles of chitin-protein fibrils (fibres) are surrounded by mineral. These results accord well with previous results on the tergite cuticle of *T. europaeus* (Seidl et al. 2011). The mineralisation at the level of chitin-protein fibrils has also been described for the cuticle of *Homarus americanus* (Nikolov et al. 2011).

Other important structural features of the crustacean cuticle are the distribution, shape and size of pore canals. The pore canal system serves not only as a transport system during cuticle mineralisation (Travis 1965, Roer 1980, Giraud-Guille 1984, Roer and Dillaman 1984) but can also fulfil mechanical tasks (Sachs et al. 2008). In contrast to some decapod species in which pore canals are devoid of mineral (Fabritius et al. 2011), the pore canals of *P. scaber* as well as *T. europaeus* are filled with mineral. This is certainly affecting the mechanical properties of the cuticle, probably leading to higher cuticle stability compared to cuticles with pore canals that contain no mineral.

Conclusion

The present paper shows that investigation of crustacean cuticle by using an improved protocol for demineralisation and fixation for TEM/STEM, the analysis of thin sections of non-demineralised cuticle, and etching of polished cuticle samples can reveal new aspects of cuticular structure and mineral distribution. Combining the results from decalcified resin embedded specimens and those from thin sections of non-decalcified cuticle has led to the demonstration of a distinct distal exocuticle that differs in the organisation of the organic matrix at the level of chitin-protein fibrils and fibres, from those in the proximal exocuticle and the whole endocuticle. Thin sectioning of non-decalcified cuticle samples in combination with low voltage STEM is also a valuable method to gain new and detailed information about the distribution of mineral and organic fibrils/fibres with high spatial resolution. In combination with TEM/STEM of resin embedded material, this method revealed details such as mineral filled pore canals and chitin-protein fibrils forming a twisted plywood structure without assembling in fibres. Etching of polished samples has the advantage that shrinkage of mineralised regions is minimised. This is of particular interest when length scales have

to be quantified with high accuracy. Furthermore, etching reveals well the orientation of fibrous organic structures that, in combination with differential etching of amorphous and crystalline mineral phases, allows for the allocation of mineral phases to specific cuticular structures.

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The postmarsupial development of *Porcellio siculoccidentalis*, with some data on reproductive biology (Crustacea, Isopoda, Oniscidea)

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Abstract

In the broader context of research on the Sicilian *Porcellio imbutus*-complex, the postmarsupial development of *Porcellio siculoccidentalis* Viglianisi, Lombardo & Caruso, 1992 was studied in detail. This research was conducted in the laboratory under controlled conditions, allowing us to follow the stages of development, from the formation of the marsupium in ovigerous females until the larval stages and development of the seventh pair of legs. The timing of developmental stages and the morphological modifications of appendages in the postmarsupial manca stages (M I–M III) are described. The manca stage M I had a duration of about one hour. Ovigerous females were collected and reared separately, and the number of parturial molts in the absence of males was counted. The results showed a maximum of four successive parturial molts. Fecundity and fertility were evaluated as the number of eggs and embryos, respectively, inside the marsupium of the ovigerous females. Both parameters were positively correlated with the size of the females. The maximum numbers of eggs and embryos in the marsupium were 113 and 141, respectively. Data describing the total number of postmarsupial mancas released per month indicated that the highest release occurred in April.

Keywords

Terrestrial isopods, postmarsupial mancas, fecundity, fertility, larvae

Introduction

Porcellio siculoccidentalis Viglianisi, Lombardo & Caruso, 1992 is a species of terrestrial isopod, belonging to the *Porcellio imbutus*-complex, whose biology is still largely unknown. It was recently described in a study (Viglianisi et al. 1992) verifying the validity of *Porcellio imbutus* Budde-Lund, 1885, a Sicilian species that is present throughout the island. This report noted that *P. imbutus* constituted four distinguishable population groups with slight but consistent morphological differences. Confirmation of these distinct groups by allozyme analysis made it necessary to consider the four groups as different species: *P. imbutus*, *P. siculoccidentalis, P. baidensis* Viglianisi, Lombardo & Caruso, 1992, and *P. hyblaeus* Viglianisi, Lombardo & Caruso, 1992. *Porcellio siculoccidentalis* can be quite easily separated from the other species by examination of the male secondary sexual characters (see Viglianisi et al. 1992). Specifically, its pleopod 1 exopodite has a rectangular internal lobe with an obliquely truncated apex, and the pereopod 7 has a rounded bump in the carpus.

During studies of the isopod fauna of Sicily, we found two large populations of *P. siculoccidentalis* from two different holm-oak woods (*Quercus ilex*), one on the Mount San Giuliano, and one on the Mount Inici, both in Trapani province (western Sicily). The population from Mount San Giuliano was found to be infected with long Mermithid nematodes that lived in the haemocoel of the isopods; some specimens were also infected with Iridovirus. To determine whether and how these symbionts modified the biological parameters of the species, we began to study the reproductive biology of the uninfected specimens of the populations found on Mount Inici and Mount San Giuliano, so as to make future comparisons with the infected specimens from Mount San Giuliano.

The aim of this report is to clarify some biological aspects of the species. In particular, we investigated the first stages of postmarsupial development and assessed the fecundity and fertility parameters. Here, we describe the postmarsupial manca stages, and the number of successive parturial molts and births after only one mating, as well as provide some data on the total number of mancas released per year.

Materials and methods

The animals were collected by hand with the aid of entomological forceps, from the litter and under stones of the holm-oak woods of Mount San Giuliano and Mount Inici, both in Trapani province (western Sicily). Sampling sites are indicated in Table 1. In the laboratory, appropriate breeding was carried out in plastic boxes of 35 × 60 cm, with soil that had been taken from the sampling sites and sterilized. The specimens were fed with slices of potatoes and carrots, and sterilized and previously moistened plane-tree (*Platanus acerifolia*) leaves. The breeding substrate was periodically moistened with vaporized water, and kept at constant temperature of 20°C (±1°C).

Table 1. Localities, WGS84 coordinates, and altitudes of the sampling sites.

Locality	WGS84 coordinates	Altitude (a.s.l.)
Monte S. Giuliano, Erice (TP)	38°2'6.92"N, 12°34'54.55"E	681 m
Monte S. Giuliano, Erice (TP)	38°2'12.30"N, 12°35'33.50"E	646 m
Monte Inici, Castel. del Golfo (TP)	38°1'1.12"N, 12°51'25.35"E	1064 m

Ovigerous females were collected from the sampling sites and were bred separately. Each female was kept in a Petri dish with a substrate made of chromatography paper, periodically moistened, and fed with slices of potatoes and carrots. Each time an ovigerous female was observed during the release of the mancas from the marsupium; the mancas were followed until the first ecdysis occurred. After that, the mancas were counted, separated from the female, and reared in different Petri dishes, using the same methods as already described.

Ovigerous females were observed daily in order to record the time of the manca release. The postmarsupial mancas were also checked daily in order to observe the molting process and record the time of each manca stage.

Fifty ovigerous females were dissected in order to analyze the marsupium content; eggs and embryos were counted (embryo stages: S14-S18, *sensu* Milatovic et al. 2010) to estimate the fecundity and fertility parameters, respectively indicated by the number of oocytes in the marsupium, and by the number of embryos in relation to the number of eggs. The number of eggs and embryos in the marsupium was related to the body length of the ovigerous femals, according to AlJetlawi and Nair (1994), that worked on similar Mediterranean biotopes.

Throughout postmarsupial development, at least ten individuals that were representative of each postmarsupial manca stage were fixed in 70% ethanol for the successive descriptions and measurements. The observations and the dissections were performed using a stereomicroscope (Leica EZ4D) and dissection forceps (Dumont & Fils No. 5). The manca stages were described following the standard procedures (Araujo et al. 2004, Brum and Araujo 2007, Sokolowicz et al. 2008), including morphological study of the appendages (antenna, antennula, maxilla, maxillula, maxillipede, pereopods, and pleopods). These parts were mounted on slides, and drawings were made using the optical microscope (Ernst Leitz Wetzlar) and a *camera lucida*. The measurements were taken using the stereomicroscope (Leica M205) equipped with a digital camera (DFC420), and using the dedicated software LES ver. 3.3.0. SEM photographs were taken with a ZEISS Evo LS 10.

Results

One hundred ovigerous females were taken in order to study their reproductive biology in the laboratory, to determine the fecundity and fertility parameters, and to describe the larval stages of the species. The body lengths of this sample ranged from 6.5

mm to 16 mm (Fig. 1). A total of 1153 specimens, including 653 females (57%) and 500 males (43%) resulted from the breeding. The births occurred in October of 2009, and between January and May of 2010, with a peak in April, during which the highest number of mancas was released (Fig. 2).

All ovigerous females showed a seasonal reproduction, with 1–4 successive parturial molts occurring before entering a non-reproductive period. The females had never successively mated with males. The times between successive parturial molts ranged

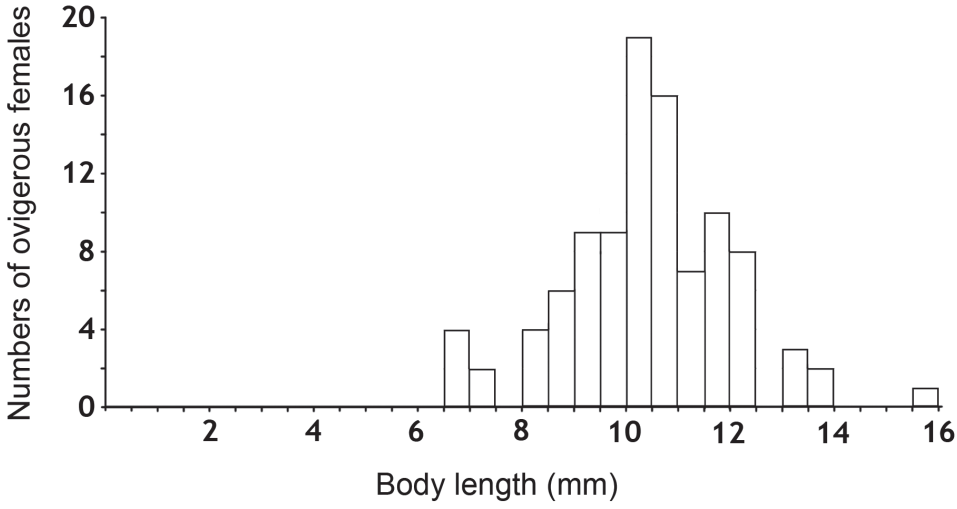

Figure 1. Distribution of body lengths of the ovigerous females used in the present study.

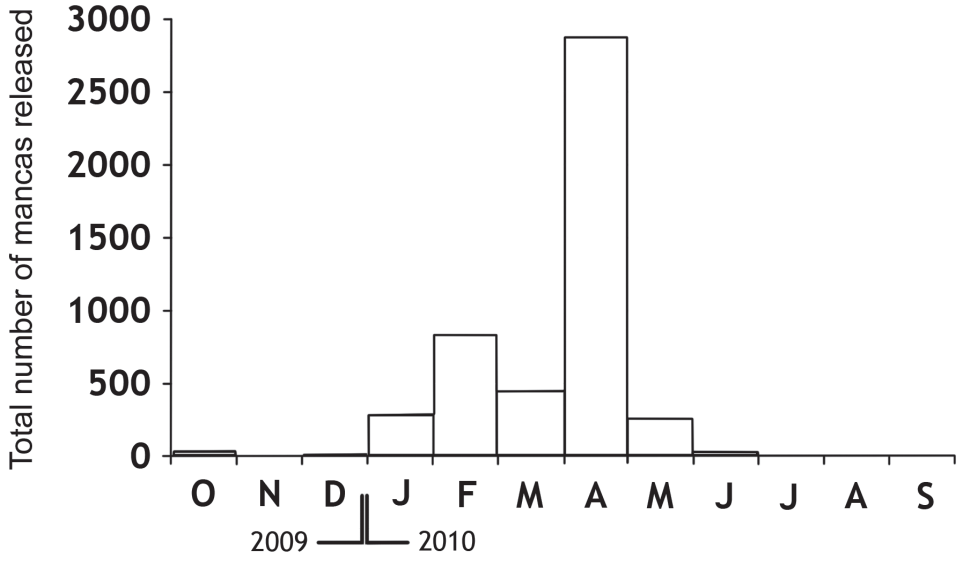

Figure 2. Total number of mancas released during the study period.

from 20 to 40 days. During the reproductive period, only one parturial molt was recorded for 39 females, two successive parturial molts for 46 females, three successive parturial molts for 12 females, and four successive parturial molts were recorded for 3 females only.

The survival rate of females after the brood release was as follows: 9% of the females died before the first brood release, 72% gave only one brood release, 16% gave two successive brood releases, and only 3% gave three successive releases of mancas.

Fecundity, estimated by the number of eggs in the marsupium, showed a positive correlation with the female size (R^2 = 0.85), varying from 22 eggs in a female with body length of 7.9 mm, to 113 eggs in a female with body length of 12.1 mm. Fertility, estimated by the number of embryos in the marsupium, also showed a positive correlation with the size of the female (R^2 = 0.93), varying from 48 embryos in a female with body length of 10.56 mm, to 141 embryos in a female with a body length of 16 mm (Fig. 3).

A positive correlation was also observed between the number of mancas released and the cephalothorax width of the females. The number of mancas released ranged from 7 for a female with cephalothorax width of 1.62 mm, to 108 for a female with cephalothorax width of 2.76 mm, with a mean value of 48 mancas per female (Fig. 4).

Porcellio siculoccidentalis showed three postmarsupial larval stages, also called manca stages (M I, M II, and M III), separated by ecdysis and characterized by the absence of the first pleopods and non-functional seventh pereopods. The main characteristics that were distinctive of each stage relate to the length of the flagellum articles, the developmental level of the pereopod 7, the number of setae of pleopods 2–5, and the number of ommatidia (Table 2).

Manca I stage (M I). The duration of this stage varied from a minimum of 57 min to a maximum of 66 min, with a mean value of 62 min. The mean body length was 1.788 mm (SD: ±0.053), with a range from 1.653 mm to 1.82 mm. The mean cephalothorax width was 0.48 mm, with a minimum of 0.35 mm and a maximum of 0.53

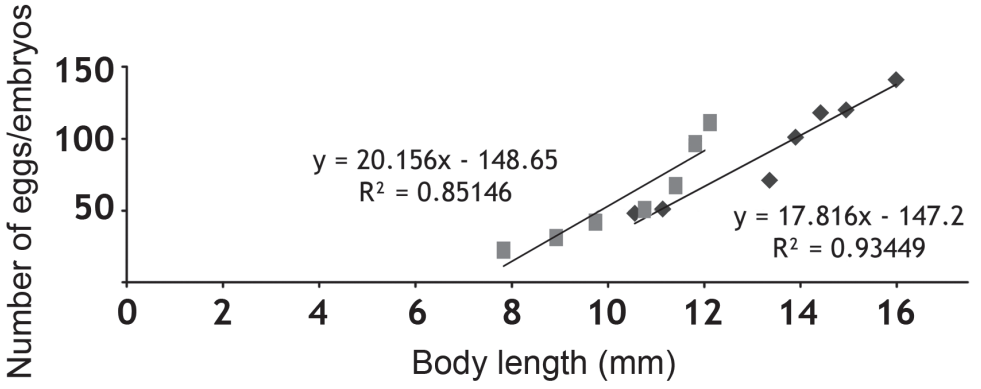

Figure 3. Correlation of the ovigerous females' body lengths with the number of eggs (■ - fecundity) and with number of embryos in the brood pouch (♦ - fertility).

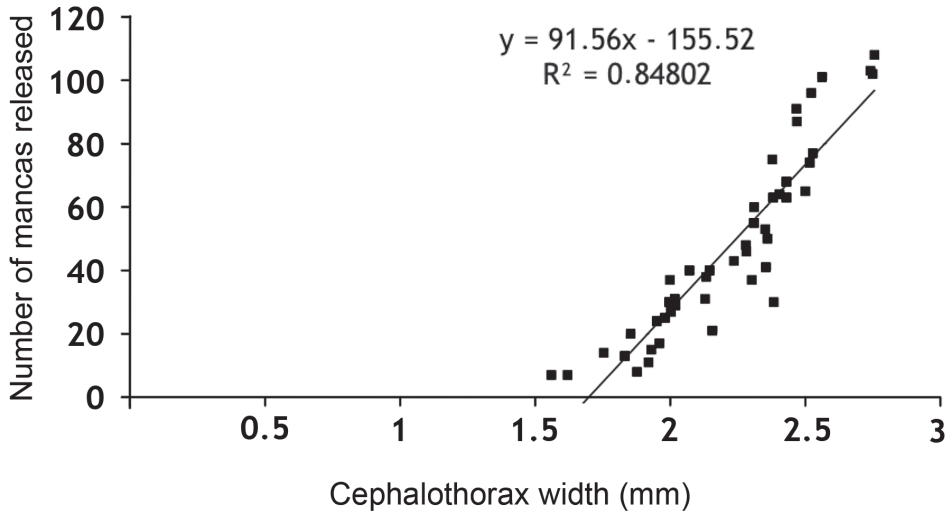

Figure 4. Correlation between cephalothorax widths of the ovigerous females with the number of mancas released.

Table 2. Summary of the main characteristics of the manca stages of *Porcellio siculoccidentalis*: ommatidia, antenna, pereopod 7, and pleopods exopodite.

Manca Stage	No. of ommatidia	Antenna: flagellum	Pereopod 7	Pleopod 2–5 exopodite
M I	4	Proximal article longer than the distal	absent	Distal margin with one seta
M II	5	Distal article longer than the proximal	folded ventrally; articles not distinguishable	Distal margin with 4–7 setae
M III	7	Distal article longer than the proximal	folded ventrally; articles distinguishable	Distal margin with 7–10 setae

mm. The first larval stage had no pigmentation, except for the ommatidia and little spots on the pereonite margins. Because of the transparency it was possible to observe the presence of food in the gut. Eyes had four ommatidia. Pereonite 7 and seventh pereopod were absent. The flagellum was bi-articulated and was as long as the fifth article and, in contrast to the other larval stages, the proximal article was twice as long as the apical (Figs 5a, 6a). Antennula showed three articles and three apical aesthetascs (Fig. 6b). Maxilla had one seta on the apical part, between the lateral and the medial lobes; five setae were present on the medial lobe (Fig. 6c). Maxillula had nine teeth (Fig. 6d). The maxillipede had the palp with apical setal tuft, and two setae in the basal region; the endite had two teeth and one apical seta (Fig. 6e). Pereopods 1–6 had few setae that were distributed generally on the lateral margins of the articles (Fig. 6f–k). Pleon was lacking the first pleopods. Pleopods 2–5 exopodite had one small seta on the distal margin (Fig. 6l–o).

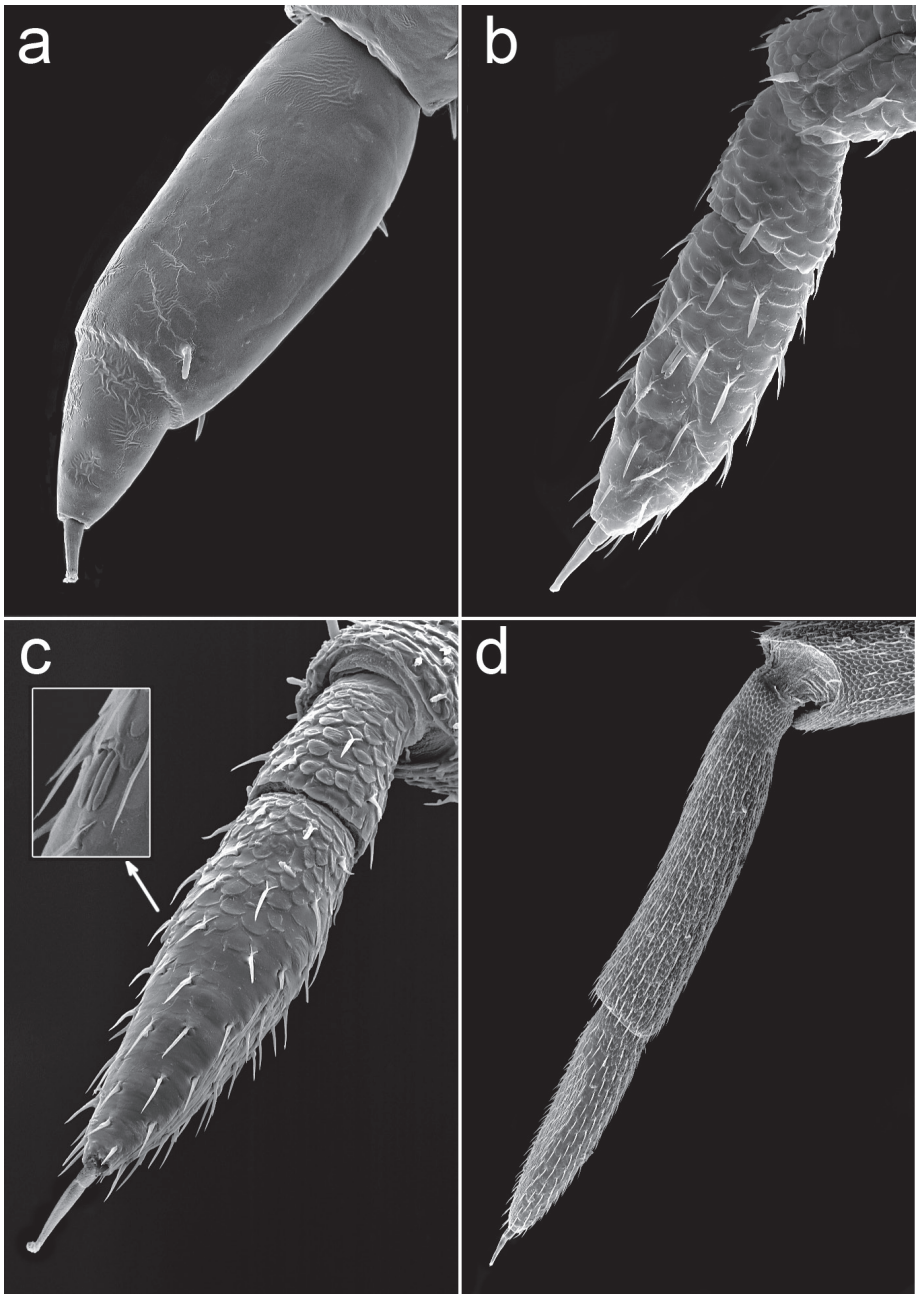

Figure 5. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Flagellum: **a** manca stage M I (500X) **b** manca stage M II (330X) **c** manca stage M III (300X) **d** adult (65X).

Manca II stage (M II). The time needed to pass from M I to M II was very short, with an average of about 62 min. The intermolt time of M II was almost 10 days, with a minimum of 8 days and a maximum of 11 days. Our M II specimens were 2.023 mm

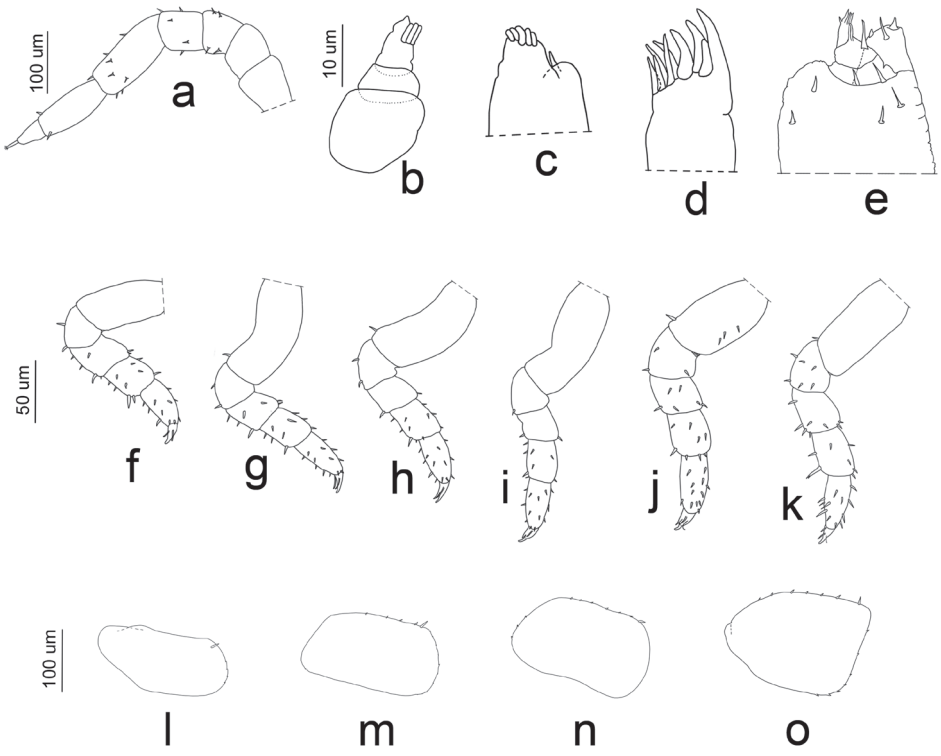

Figure 6. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Manca stage M I: **a** antenna **b** antennula **c** maxilla **d** maxillula **e** maxillipede **f** pereopod 1 **g** pereopod 2 **h** pereopod 3 **i** pereopod 4 **j** pereopod 5 **k** pereopod 6 **l** pleopod 2 exopodite **m** pleopod 3 exopodite **n** pleopod 4 exopodite **o** pleopod 5 exopodite.

long on average (SD: ±0.098), with a minimum value of 1.63 mm and a maximum of 2.12 mm. Cephalothorax mean width was 0.62 mm, with a minimum of 0.59 mm and a maximum of 0.65 mm. Pigmentation on the pereionite margins was stronger than in M I and appeared also on the cephalothorax. Eyes had five ommatidia. Antenna article lengths showed an inversion of those of flagellum, with the distal article twice the length of the proximal (Fig. 5b, 7a). Antennula was similar to in the M I stage (Fig. 7b). Maxilla showed a high number of setae only on one lobe; the two lobes were of the same size (Fig. 7c). Maxillula had lateral setal tuft and nine teeth, three of them were bifid (Fig. 7d). The maxillipede showed palp with one apical setal tuft, and two long apical setae with one in the basal position; endite had three teeth and one apical seta (Fig. 7e). Pereonite 7 appeared. Pereopods 1–6 had stronger and more numerous setae than in the M I stage (Fig. 7f–k); pereopod 7 began to develop, but it was not possible to distinguish articles (Fig. 8a). Pleopod 1 was absent; pleopod 2 had one seta; and pleopods 3–5 had five, seven, and four setae, respectively, on the distal margin (Fig. 7l–o).

Manca III stage (M III). Intermolt duration of this stage was on average 11 days, with a minimum of 10 days and a maximum of 12 days. Our M III specimens were

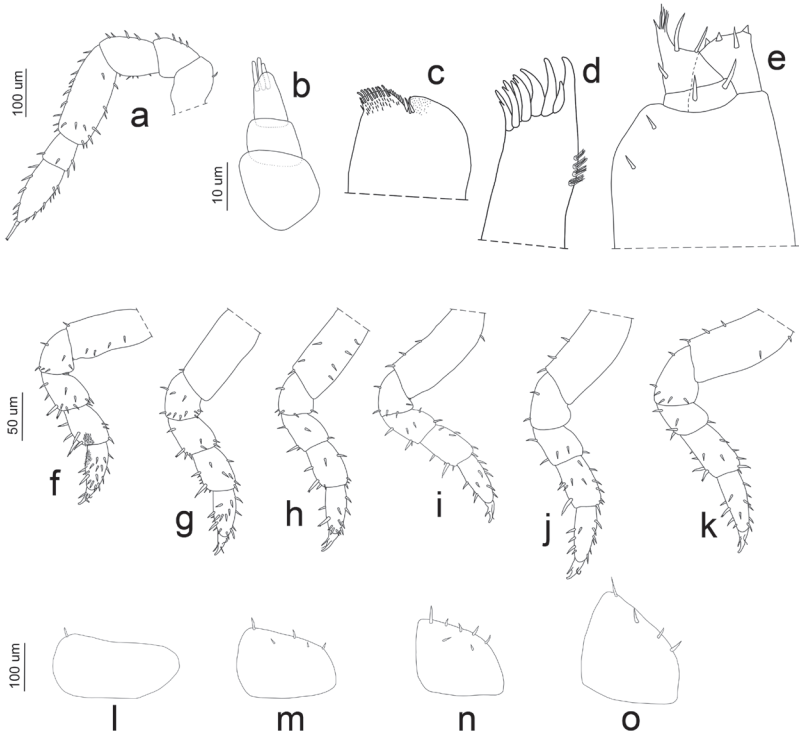

Figure 7. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Manca stage M II: **a** antenna **b** antennula **c** maxilla **d** maxillula **e** maxillipede **f** pereopod 1 **g** pereopod 2 **h** pereopod 3 **i** pereopod 4 **j** pereopod 5 **k** pereopod 6 **l** pleopod 2 exopodite **m** pleopod 3 exopodite **n** pleopod 4 exopodite **o** pleopod 5 exopodite.

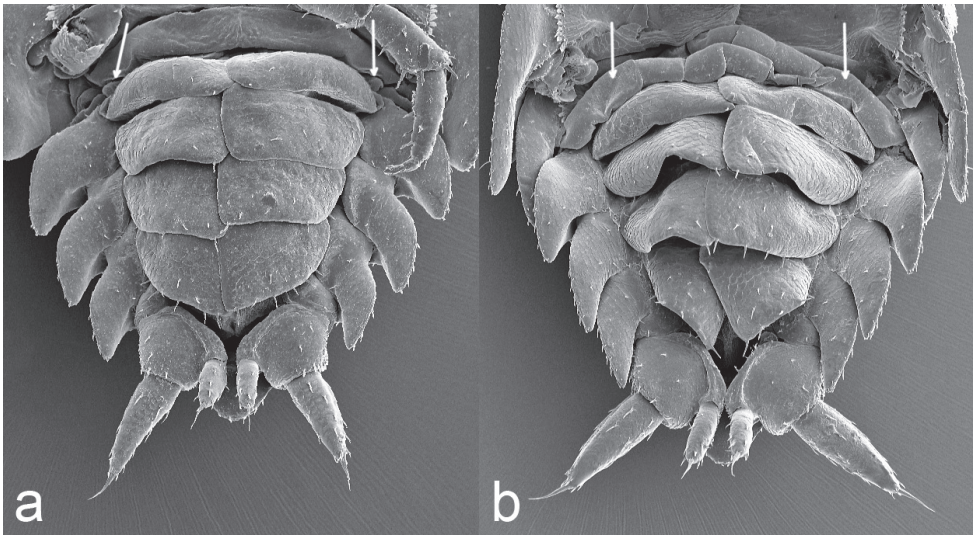

Figure 8. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Pleon (ventral vision) and pereopods 7 (indicated with white arrows): **a** manca stage M II (80X) **b** manca stage M III (80×).

2.465 mm long on average (SD: ±0.149), with a minimum of 2.2 mm and a maximum of 2.633 mm. Cephalothorax width varied from a minimum of 0.66 mm to a maximum of 0.796 mm, with a mean of 0.72 mm. A light brown pigmentation could be observed on the whole body dorsal surface. Eyes consisted of seven ommatidia. Antennae were similar as in the M II stage (Fig. 9a), but the distal article of the flagellum was more than twice the length of the proximal (Fig. 5c). Antennula had four aesthetascs (Fig. 9b). Maxilla had a higher number of setae on the lobe than in the M II stage (Fig. 9c). Maxillula was similar to the M II stage, but with numerous lateral setal tufts (Fig. 9d). The maxillipede was similar to the M II stage (Fig. 9e). Pereonite 7 was clearly visible. Pereopods 1–6 were bigger and more robust with a higher number of setae than in the M II stage (Fig. 9f–k). Pereopod 7 was visibly folded in the ventral position

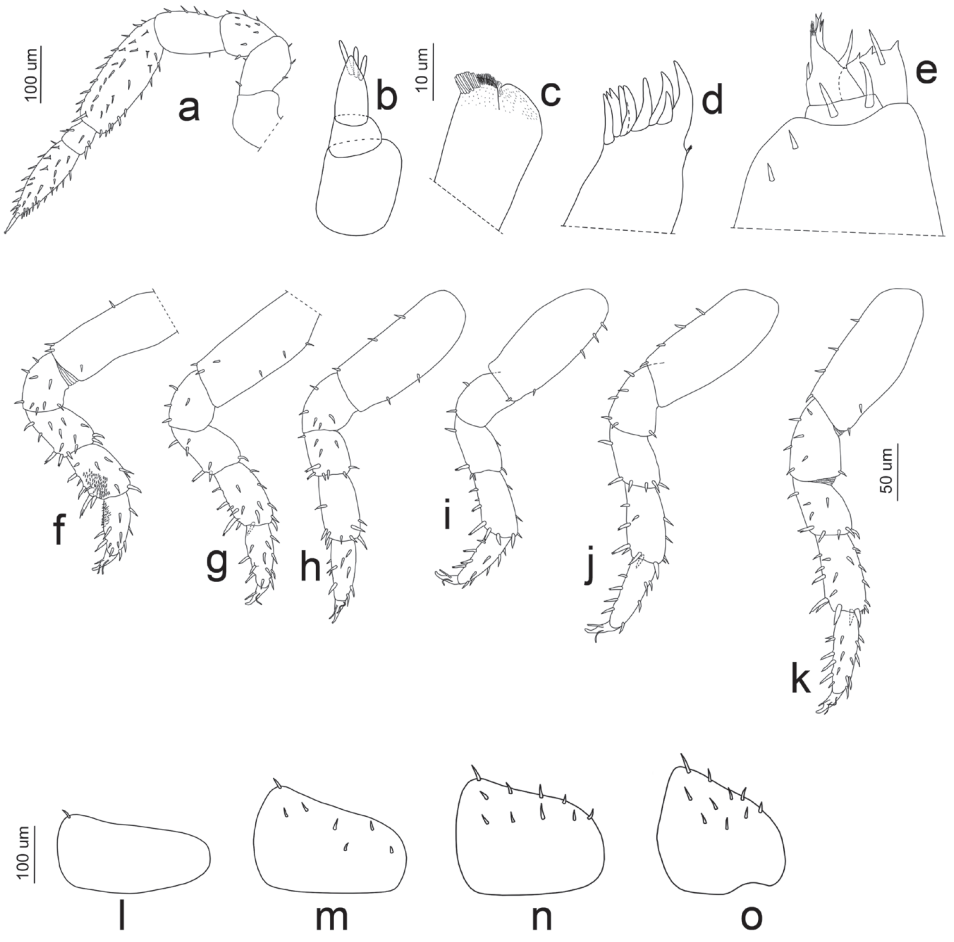

Figure 9. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Manca stage M III: **a** antenna **b** antennula **c** maxilla **d** maxillula **e** maxillipede **f** pereopod 1 **g** pereopod 2 **h** pereopod 3 **i** pereopod 4 **j** pereopod 5 **k** pereopod 6 **l** pleopod 2 exopodite **m** pleopod 3 exopodite **n** pleopod 4 exopodite **o** pleopod 5 exopodite.

Table 3. Mean values (±DS) of cephalothorax width, body length, and duration of each manca stage of *Porcellio siculoccidentalis*.

Manca stage	Cephalothorax width (mm)	Body length (mm)	Duration
M I	0.48 ± 0.018	1.788 ± 0.053	62 ± 8.49 min
M II	0.62 ± 0.019	2.023 ± 0.098	10 ± 0.94 days
M III	0.72 ± 0.04	2.465 ± 0.149	11.7 ± 1.45 days

and the articles could be distinguished (Fig. 8b). Pleopod 1 was absent; pleopod 2 had one seta; pleopods 3 had seven setae, while pleopods 4–5 had ten setae (Fig. 9l–o).

Mean values of cephalothorax width, body length, and duration of the three stages are reported in Table 3.

Discussion and conclusions

Recently, many papers have described different aspects of the reproductive biology of terrestrial isopods. They cover some topics relating to the female reproductive system (Warburg 2011a), gestation (ovaries, oocytes), incubation, and embryogenesis taking place in the marsupium (Milatovic et al. 2010; Appel et al. 2011). Other recent papers have reviewed some aspects of post-parturial events concerning breeding periods, strategies and patterns, parturition, and size and number of broods (Warburg 2011b). Some other related ecological subjects concerning the adults have also been recently reviewed (Knight 2008; Hornung 2011). Despite this wealth of newly published data, there are few descriptions of the postmarsupial manca stages of terrestrial isopods; these are very important steps that bring a species to the juvenile stages and then to the adult stage. The present study provides a complete description of the postmarsupial manca stages.

In this study, fecundity and fertility were evaluated as the number of eggs and embryos, respectively, inside the marsupium of the ovigerous females. Both parameters were positively correlated with the size of the females. Comparable data were previously reported for *Armadillo officinalis* Duméril, 1816, from Libya (AlJetlawi and Nair 1994). Data from females with similar body lengths showed higher fecundity values than fertility values, indicating that not all of the eggs developed into embryos.

Data regarding the number of manca released were similar to those that have been reported for *Armadillo officinalis* (AlJetlawi and Nair 1994) from Libya, and for some neotropical species of Oniscidea, including *Atlantoscia floridana* (Van Name, 1940), *Benthana cairensis* Sokolowicz, Araujo & Boelter, 2008 (Sokolowicz et al. 2008), *Balloniscus glaber* (Araujo and Zardo, 1996), and *Balloniscus sellowii* (Brandt, 1833) (Quadros et al. 2008).

In the present study, we observed that after release from the marsupium, the stage M I mancas were very thin and fragile and it was possible to observe sternal calcium deposits (Fig. 10) that mark the premolt stage (Zidar et al. 1998). This suggests that the M I mancas leave the marsupium in a premolt stage. For different terrestrial isopod spe-

Figure 10. *Porcellio siculoccidentalis* Viglianisi et al., 1992. Specimens of manca stage M I, after the release from the brood pouch (arrow indicate calcium deposits).

cies, Heeley (1941, 1942) first defined this stage as being characterized by incomplete segmentation, and a thin body with no evident pigmentation, characteristics that were also found in the manca I stage of *P. siculoccidentalis*. This stage was also reported for *Atlantoscia floridana* (Araujo et al. 2004) and *Benthana cairensis* (Sokolowicz et al. 2008). The authors suggested that stage M I represents the last marsupial stage, when the animals did not molt or eat. In *P. siculoccidentalis*, the first postmarsupial manca stage had a duration of only one hour, which is the shortest among the postmarsupial mancas previously described in the literature. For *Benthana cairensis*, the M I stage is 4-hours long, and the duration in other species varies from 12 hours for *Atlantoscia floridana* (Araujo et al. 2004), to 19 hours for *Porcellio dilatatus* Brandt, 1833 (Brum and Araujo 2007), to 48 hours for *Hemilepistus reaumurii* (Milne-Edwards, 1840) (Kacem-Lachkar 1997).

One observation from this study that, to our knowledge, has never been previously reported regards the mancas at the moment of the release from the marsupium. We observed that the newly released specimens at stage M I stayed under or near the female without moving until the first molt was completed. Only after molting (at stage M II) mancas started to move, exploring the environment and searching for food. In *P. siculoccidentalis*, the mancas ate their exuviae after the first molting, as has been

reported for other species (Helley 1941, Araujo et al. 2004, Brum and Araujo 2007, Sokolowicz et al. 2008).

One remarkable characteristic of stage M I regards the antennal flagellum. In this stage, the proximal article was longer than the distal; however, in the following manca stages, the proportions changed, and the distal article was longer than the proximal. This change is common during the developmental stages in different species belonging to various families of terrestrial isopods (Verhoeff 1920, Heeley 1941, Araujo et al. 2004, Brum and Araujo 2007). In *P. siculoccidentalis*, the proportion changed again in the adults, where the distal article was shorter than the proximal (Fig. 5d).

During stage M II, the mancas were observed to feed normally, showing stronger and robust exoskeleton and pereopods; moreover, the seventh pereonite and pereopods developed, although the articles could not yet be distinguished. The premolt stage can be detected observing the calcium deposits in the pereon sternites (Zidar et al. 1998).

Stage M III was similar to stage M II with only a few differences. The seventh pereonite was larger than in M II. Additionally, the seventh pereopods were present in M III and showed all articles, although they were in a non-functional condition and remained folded ventrally under the body.

As previously mentioned, there are few papers in the literature that describe the manca stages of terrestrial isopods. However, the work already published clearly indicates that the major differences among the species relate to modifications of the appendages of the cephalothorax, pereon, and pleon (Verhoeff 1920, Haddad 1982, Araujo et al. 2004, Brum and Araujo 2007, Sokolowicz et al. 2008).

Here, three postmarsupial larval stages are described for *P. siculoccidentalis*. After the third molt, the animals pass to the juvenile stages. The seventh pereopods become perfectly functional and the differentiation of the first pleopods begins. Lastly, the beginning of sexual differentiation can be observed.

Finally, in this study on *P. siculoccidentalis*, we found no differences between the populations from Mount San Giuliano and Mount Inici. Specimens of both populations showed identical morphology of the appendages, and similar values were obtained regarding size, survival rate, number of manca released, fecundity, and fertility. No other differences were found on the reproductive biology of the two populations.

Comparative data obtained from the infected specimens from Mount San Giuliano will provide new information about any modifications caused by the presence of nematodes and/or Iridovirus infection. This will be a future task and a natural continuation of the present work.

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Biomineralizations: insights and prospects from crustaceans

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Abstract

For growing, crustaceans have to molt cyclically because of the presence of a rigid exoskeleton. Most of the crustaceans harden their cuticle not only by sclerotization, like all the arthropods, but also by calcification. All the physiology of crustaceans, including the calcification process, is then linked to molting cycles. This means for these animals to find regularly a source of calcium ions quickly available just after ecdysis. The sources of calcium used are diverse, ranging from the environment where the animals live to endogenous calcium deposits cyclically elaborated by some of them. As a result, crustaceans are submitted to an important and energetically demanding calcium turnover throughout their life. The mineralization process occurs by precipitation of calcium carbonate within an organic matrix network of chitin-proteins fibers. Both crystalline and stabilized amorphous polymorphs of calcium carbonate are found in crustacean biominerals. Furthermore, Crustacea is the only phylum of animals able to elaborate and resorb periodically calcified structures. Notably for these two previous reasons, crustaceans are more and more extensively studied and considered as models of choice in the biomineralization research area.

Keywords

ACC, amorphous calcium carbonate, biomineralization, calcification, calcium storage, cuticle, organic matrix

"Crustaceans are the champions of mineral mobilization and deposition in the animal kingdom"

Lowenstam and Weiner 1989

Introduction

Biomineralization corresponds to the process of mineralized structures formation by living organisms. This word designates also the elaborated mineralized structure itself. This phenomenon, which appeared firstly in Eubacteria and Archea as a biologically-induced process (Lowenstam 1981), is widespread in the Metazoa kingdom as a biologically-controlled process mediated by an organic matrix (Mann 1983), also termed template-directed mineralization (Pouget et al. 2009). The first major function of this process is the hardening of a skeleton, a structure that provides support for muscles and protection against environmental pressures (Lowenstam and Weiner 1989, Simkiss and Wilbur 1989). The fossil species, discovered so far, revealed that the first calcified metazoan exoskeletons appeared probably at the end of the Precambrian period, during the Proterozoic (Kirschvink and Hagadorn 2000, Knoll 2004). The oldest known crustacean is dating 520 million years, discovered in the Early Cambrian Maotianshan Shale Lagerstätte, in China (Chen et al. 2001). The reasons for the appearance and selection of the biomineralization process during evolution remain speculative but several hypotheses have been mentioned including protection against predators. The adverse evolution of the ionic composition of the primitive ocean, notably the calcium ion concentration, was also raised.

The biomineralization process is well developed in several taxa, including Crustacea as one of the most outstanding group in this respect. As a consequence of the presence of a rigid exoskeleton, the growth and the whole physiology of these animals are linked to molting cycles characterized by the complete renewal of the exoskeleton during each molting. All of the arthropods harden their new cuticle by a process called sclerotization (protein-polysaccharide and protein-protein cross-linking by the way of quinonoid-sclerotizing agents) and, in addition, most of the crustaceans proceed furthermore by calcification.

The major source of calcium used for exoskeleton calcification is exogenous: the water in which most crustaceans live. In seawater, calcium concentration is very high but many species also live in freshwater or on land, where the availability of calcium at ecdysis may be low or even absent. Therefore crustaceans have developed different strategies to solve the problem of calcification, in particular by storing calcium during the premolt period (Graf 1978, Greenaway 1985), a phenomenon especially well developed in terrestrial species of amphipods, isopods and decapods. The odd thing is that calcium storage is also found in some aquatic species. The food (including sometimes the exuviae), a possible source of calcium ions, represents a minor contribution to the cuticle calcification, whatever the way of life of the animal considered.

Next, crustaceans are particularly interesting because of their active calcium metabolism and their ability to form and resorb cyclically (more or less partially) not only an external calcified structure but also, in many species, calcium storage biomineralizations.

Another characteristic of calcium metabolism in crustaceans are the calcium-transporting epithelia very similar to some vertebrate ones, and in this way they represent also good models to understand how they function. Calcium pumps and enzymatic

Figure 1. Adult specimen of the freshwater red claw crayfish, *Cherax quadricarinatus*, just after molting (at left the exuviae).

systems, similar to those associated with vertebrate calcium-transporting epithelia, have been evidenced in the cuticular epidermis and the calcium storage epithelium. Furthermore crustaceans are convenient models for studying the hormonal regulation of the calcium turnover with the possible involvement of vertebrate-type hormones such as calcitonin/CGRP and vitamin D presumably evolving from invertebrate counterparts (McWhinnie et al. 1969, Greenaway 1985, Fingerman et al. 1993, Luquet and Marin 2004).

Molting cycle, cuticle and calcification

In crustaceans, a cellular hypodermis, which underlies the calcified cuticle also called carapace, is responsible for the complete synthesis of the exoskeleton. This cuticle com-

prises four main layers, from the external to the innermost layer: the epicuticle, the exocuticle, the endocuticle and the membranous layer. Calcification of this cuticle has been particularly well studied in Decapoda (Travis 1963, Roer 1980, Greenaway 1985). Except for the arthrodial cuticle present at the joints of appendages and at the base of gills and setae, for the cuticle covering the gills and the gut, and for the membranous layer of the exoskeleton, the three other cuticlar layers are more or less mineralized. The process occurs essentially by precipitation of calcium carbonate into an organic matrix network of chitin-protein fibers arranged in a twisted plywood and honeycomb-like structure (Bouligand 1972, Giraud-Guille et al. 2004). Calcification of the carapace takes place at different sites within the cuticle: around chitin-protein fibers, at the level of interprismatic septa, around and within pore-canals formed by cytoplasmic extensions of hypodermal cells (Roer and Dillaman 1984, Giraud-Guille 1984a, 1984b, Compère et al. 1992, 1993, Giraud-Guille et al. 2004, Romano et al. 2007). Until recently it was thought that the calcification of the decapod cuticle occurred mainly in a crystalline form (calcite or Mg-calcite). Some recent investigations confirmed the presence of a crystalline polymorph but revealed that ACC and ACP are also present in various amounts, depending on the species concerned (Dillaman et al. 2005, Romano et al. 2007). For example, by following the early hours of the carapace calcification of the blue crab, *Callinectes sapidus*, Dillaman and co-workers (2005) evidenced that calcium carbonate is first deposited as ACC, which then transforms into calcite. Other considerations came from the extensive study of the American lobster, *Homarus americanus*, cuticle (Sachs et al. 2006, Romano et al. 2007, Raabe et al. 2008, Al-Sawalmih et al. 2008, 2009, Fabritius et al. 2009, Nikolov et al. 2010, 2011). They defined 7 hierarchical levels of cuticle organization, from the polymerization of N-acetyl-glucosamine (level I) to the finished product, the complete calcified cuticle (level VII). Level IV corresponds to the deposition of calcium carbonate around chitin-protein nanofibers. On the other hand, they demonstrate that only the outer part of the exocuticle of the American lobster is calcified with calcite/Mg-calcite, whereas the rest of the exocuticle as well as the endocuticle are completely calcified with ACC and, in a lesser extent, with ACP. They also analyzed the relations referring to the multiple levels of the cuticle complex structure, from the nanoscale to the macroscopic level, and its remarkable mechanical properties as an inspiration source for biomimetic materials research.

Until recently, the structure and calcification of the cuticle in isopods have been poorly investigated (Price and Holdich 1980a, 1980b, Wood and Russell 1987, Štrus and Compère 1996). Recent works have shown that the different layers of isopod cuticle could be more or less and irregularly calcified and that the composition and distribution of the mineral could vary considerably from the decapods (Becker et al. 2005, Hild et al. 2008, 2009, Seidl et al. 2011, Neues et al. 2007, 2011). If amorphous calcium carbonate has been demonstrated to be transitorily present as precursor of a crystalline polymorph (calcite essentially), similarly to decapods, stabilized ACC and in a lesser extent ACP have been evidenced as components of isopod cuticle. For example, in *Armadillidium vulgare* and *Porcellio scaber* (Neues et al. 2007, Hild et al. 2008), the epicuticle and the membranous layers are not mineralized, the exocuticle

contains both Mg-calcite and ACC/ACP whereas the endocuticle is only calcified with ACC. The thickness of the endocuticle appears correlated with the behavior of these terrestrial crustaceans for avoiding a possible environmental danger: *P. scaber* avoids predation by running away, which requires a thin and flexible cuticle (with 16% Mg-calcite, 38% ACC and 12% ACP), whereas *A. vulgare* avoids predation by rolling into a sphere and thus possesses a thicker and more mineralized cuticle (with 12% Mg-calcite, 59% ACC and 11% ACP). Nevertheless a comparative study performed on 4 marine and 6 terrestrial isopod species revealed a great variation of the composition, more pronounced in the marine species even with similar habitats and behaviors: 12–20% Mg-Calcite, 38–59% ACC and 0–14% ACP for the terrestrial species *versus* 4–58% Mg-calcite, 17–60% ACC and only 0–3% ACP for the marine species.

Finally, the cuticle of *Ligia italica* was used as a model of matrix-mediated calcification in a comparative study using also calcite deposits generated by *Synechoccocus* cyanobacteria as a biologically-induced calcification model. As previously shown for growth zones of bones (Carlisle 1970, Landis et al. 1986), the authors demonstrate that silicon, in the form of amorphous oligomerized silicic acid, is involved in the early steps of calcification at nucleation sites, serving as intermediary between polysaccharide-protein complexes and inorganic ions catalyzing the precipitation of a mineral phase (Matsko et al. 2011).

While the presence of ACC as the main polymorph in calcium storage deposits can be easily understood (see below), the presence of metastable ACC at the level of the cuticle is more surprising. Nevertheless, some advantages of the presence of stable amorphous minerals in cuticles are shape flexibility, plasticity, as well as optimization of strenght and toughness (Ali-Sawalmih et al. 2009). Another interesting feature of ACC is that it enables easier calcium mobilization, useful for the partial decalcification of the old cuticle in every premolt as well as for cuticle repairing in intermolt. On the other hand, it has been suggested for isopods that the structure and mineralization of the cuticle can be related to the ecophysiology of the animals considered.

From the structural-mineral point of view, the presence of different polymorphs of calcium carbonate within the crustacean cuticles is tightly linked to the presence of specific matrix molecules.

Some molecules, identified as cuticular proteins, have been characterized, the precise function of which is not well elucidated. Some of them, possessing a Rebers-Riddiford domain in their sequence, a hallmark of their chitin-binding ability (Rebers and Willis 2001), are probably involved in the formation of the organic network serving as template for the precipitation of calcium carbonate. An 18-residue domain, also called cuticle_1 domain, has been found in proteins belonging exclusively to hard cuticle. They are suspected to play a role in the regulation of calcite crystal growth (Kragh et al. 1997, Nousiainen et al. 1998, Andersen 1999).

Other proteins, with *in vitro* calcium-binding ability or which interact with $CaCO_3$ formation, are also thought to play an active role in the *in vivo* $CaCO_3$ precipitation process. Among them are DD4/crustocalcin from *Penaeus monodon* (Endo et al. 2000, 2004), CAP-1 and CAP-2 from the crayfish *Procambarus clarkii* (Inoue et al. 2001, 2003,

2004, 2007, Sugawara et al. 2006,Yamamoto et al. 2008) and more recently Casp-2 from the blue crab *Callinectes sapidus* (Inoue et al. 2008) but their real *in vivo* functions remain speculative. Other technical approaches have been used recently allowing the simultaneous characterization of multiple transcripts encoding putative cuticular proteins. First, an EST database was produced from two cDNA libraries prepared from the gill and the hypodermis of the blue crab, *Callinectes sapidus* (Coblentz et al. 2006). By using 3 different strategies for screening this database, 73 transcripts were suggested to code for cuticular proteins (Faircloth and Shafer 2007). Efforts are currently made for obtaining the complete sequence of these transcripts and for determining if they encode calcified cuticle proteins versus arthrodial membrane proteins (Wynn and Shafer 2005, Shafer et al. 2006, Faircloth and Shafer 2007). The second approach aimed to develop a cDNA microarray chip for *Portunus pelagicus* for generating expression profiles of genes involved in the cuticle formation. Twenty-one differentially expressed transcripts (up-regulated in postmolt) encoding cuticular proteins were isolated (Kuballa et al. 2007). Blast analyses were performed against cuticleDB, a database comprising all the proteins of Arthropod cuticle identified so far (Magkrioti et al. 2004). The comparison was made on the base of the presence of specific domains. Thirteen of these 21 transcripts contain the cuticle_1 domain specific for hard cuticle, 4 contain a variant of the RR domain (chitin_bindin_4) found in both calcified and uncalcified cuticle and 4 possess a domain called Pfam B 109992 found associated to a cuticular protein, CPCP1876, from the rock crab *Cancer pagurus*. In another study using the same approach, Kuballa and Elizur (2008) focused on transcripts possibly associated with mineralization and sclerotization of cuticle organic matrix. More particularly, they suggested that, because of their affinity for glycoproteins, C-type lectin receptors and a mannose-binding protein (MBP1) could be involved in the regulation of calcification by two alternative pathways involving glycosylation and deglycosylation events of cuticle proteins linked to conformational changes. They are also thought to play a role in the activation of the phenoloxidase pathway. Finally, by generating two cDNAs libraries, one from the whole body, the other from specific organs (brain, eystalks, mandibular organ, Y-organs) 556 clones were sequenced among which 14% encoding cuticular proteins (Kuballa et al. 2011). According to the cuticleDB nomenclature (Magkrioti et al. 2004) cuticular proteins up-regulated in postmolt were identified: CUT proteins (CUT1 to CUT8, CUT12 and CUT13), the DB1, DB2 and DB3 proteins, the CB3 and CB4 proteins, the VR2 and VR3-like proteins, the DBM protein. More curiously, a transcript encoding the gastrolith GAP65 protein was also found suggesting first that this protein is not specific for the gastrolith disc and second that this protein is probably involved in the calcification of the cuticle, based on its role in gastrolith formation (Shechter et al. 2008; see also below the paragraph untitled "Calcium storage, In Decapoda").

Calcium storage

Except for the Copepoda and Cirripedia (Maxillopoda), calcium storage is a process commonly found in the other groups of crustaceans. The sites of storage as well as the

morphologies of the storage structures are very diversified (Graf 1978, Luquet and Marin 2004). Nevertheless, it seems that a general feature is the storage of calcium carbonate in an amorphous polymorph. The stored calcium must be quickly available after ecdysis and the amorphous polymorph of calcium carbonate (ACC) is the most compatible with this function. Chemical inorganic ACC is a metastable polymorph, which transforms immediately into a crystalline polymorph, calcite being the most stable. Even though biogenic ACC is stabilized in time by matrix molecules, it remains easily mobilizable (Addadi et al. 2003).

In Amphipoda (Malacostraca: Peracarida)

The most extensively studied model is the semiterrestrial talitrid amphipod *Orchestia cavimana*. This animal cyclically stores calcium in two diverticula of the midgut, called posterior ceca (PC), in the form of calcareous concretions (Figure 2; Graf and Meyran 1983, 1985, Meyran et al. 1984, 1986).

The storage organs are composed of a one-layered epithelium forming tubules, which are proximally connected to the midgut and distally blind-ended. During the premolt period, calcium originating from the present cuticle is transported in an ionized form and is precipitated in the PC lumen within an organic matrix synthesized by the PC cells (Figure 2C). Concretions are formed by addition of successive concentric layers of organic matrix, within which calcium carbonate is precipitated as the amorphous polymorph (Raz et al. 2002). After ecdysis, calcium resorption occurs by successive generations of 1 μm diameter calcified spherules that form at the apical part of the PC epithelium and dissolve at the basal part of the extracellular PC network. The storage proceeds exponentially during a 16-day mean period for an adult specimen concomitant with the partial demineralization of the cuticle, whereas dissolution of concretions is performed in less than 48 h. The stored calcium represents 60% of the calcium necessary for the complete calcification of each new brand cuticle.

To understand the formation of biomineralized structures, the characterization of the molecular components of the organic matrix is of first interest. Biochemical and molecular biology techniques were used to characterize proteinaceous components of the matrix. One peculiar protein, named Orchestin, has been well characterized and completely sequenced. This phosphorylated calcium-binding protein is probably responsible for the precipitation of calcium carbonate within the storage organs in premolt as well as for the formation of the calcium resorption spherules in postmolt (Testenière et al. 2002, Hecker et al. 2003, 2004). Orchestin is also thought to be involved in the determination of the amorphous calcium carbonate (ACC) polymorph, in cooperation with other matrix molecules (Hecker et al. 2004).

Other interesting amphipod models are troglobites of the genus *Niphargus*. They are able to store calcium in posterior ceca as amorphous calcium carbonate concretions, similarly to *Orchestia* amphipods, but also as rhomboedric crystalline structures (probably calcitic) within the gut (Graf 1975).

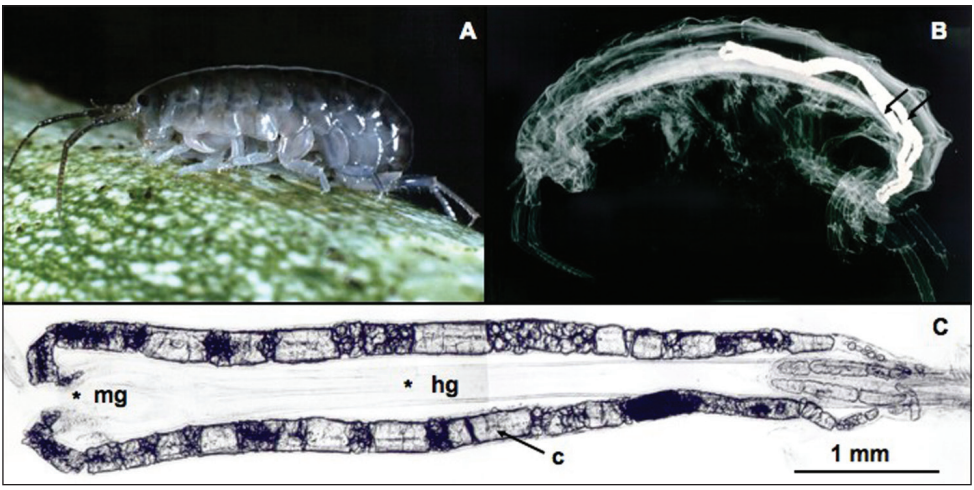

Figure 2. Calcium storage in the semiterrestrial amphipod, *Orchestia cavimana*. **A** Adult male specimen (2-cm long) **B** Radiography of an adult specimen just after ecdysis (electron-dense stored calcium is well visible in 2 diverticula of the midgut; arrows) **C** Calcium is stored as calcareous concretions in paired posterior ceca. c: concretion, hg: hindgut, mg: midgut.

In Isopoda (Malacostraca: Peracarida)

Isopods possess a particular biphasic mode of molting: they shed first the posterior half of their cuticle, then the anterior part (Messner 1965, Steel 1993, Ziegler 1994). During the premolt period, oniscid isopods elaborate calcified deposits in the four anterior sternites between the cuticle and the hypodermis. The formation of such sternal plates has been particularly well studied in the woodlouse, *Porcellio scaber* (Ziegler 1994, 1997, Fabritius and Ziegler 2003, Ziegler et al. 2005). Some results were also obtained from isopods of the genus *Oniscus* (Steel 1993), *Ligidium* (Ziegler and Miller 1997, Glötzner and Ziegler 2000) and *Ligia* (Numanoi 1934, Ziegler and Miller 1997, Glötzner and Ziegler 2000, Štrus and Blejec 2001, Ziegler et al. 2007).

The calcified storage structures are composed of amorphous hydrated calcium carbonate precipitated within an organic matrix of spherules (Ziegler 1994, Ziegler and Miller 1997, Becker et al. 2003, Ziegler et al. 2005).

The formation and resorption of these sternal plates seem closely related to the biphasic molting cycle of these crustaceans. The calcium deposits, fully developed before ecdysis of the posterior part, are completely resorbed before ecdysis of the anterior part. It has been suggested that the calcium, stored as calcospherules in the edysial space, is used to calcify the posterior cuticle (Steel 1993, Štrus and Compère 1996, Ziegler and Merz 1999, Ziegler and Scholz 1997). After ecdysis of the anterior cuticle, the stored calcium is resorbed by the epidermis and transported until the cuticle through the haemolymph in a ionic form (Ziegler and Scholz 1997, Ziegler et al. 2005, 2007).

In Decapoda (Malacostraca: Eucarida)

The order Decapoda represents the largest group of crustaceans living on land as well as in water, and storage strategies are well developed and very diversified in decapods.

For calcifying the cuticle, calcium ions are translocated from an endogenous or exogenous source through the haemolymph. In some species this medium is used as a storage site. In the freshwater/land crab, *Holthuisana transversa*, the haemolymph has been observed to contain small-size calcified granules representing a way of transporting a great amount of calcium in a short period while avoiding toxification (Sparkes and Greenaway 1984).

Hepatopancreas is also used by some crabs like *Cancer pagurus*, *Carcinus maenas*, *Callinectes sapidus* and *Paratelphusa hydrodomous* as a storage organ where calcified granules of calcium phosphate have been found, also considered by some authors as playing a role in metal detoxication processes (Adiyodi 1969, Becker et al. 1974, Guary and Negrel 1981). This was also evidenced more recently in the land crab *Ucides cordatus* (Corrêa et al. 2002, 2009).

Finally some decapods store calcium in their cardiac stomach wall between the one-layered epithelium and a cuticle as so-called gastroliths (Travis 1960, 1963). They appear as paired semi-spherical structures in lobsters and crayfishes (Figure 3A) and as four more irregular deposits in gecarcinid land crabs. After acidic decalcification, we observed an important network of concentric and transversal micro- and nanofibers of organic matrix forming meshes of different sizes (Figure 3B and C) within which calcium carbonate is precipitated as nanospheres, as generally found in all the ACC biomineralized structures (Figure 3B).

Four organic matrix proteins from gastroliths have been well characterized and sequenced so far. The first one, named GAMP, was obtained from the crayfish *Procambarus clarkii* (Ishii et al. 1996, 1998). More recently GAP65, GAP10 and crustacyanin-A2 subunit were obtained from the Australian red claw crayfish, *Cherax quadricarinatus* (Shechter et al. 2008, Luquet et al. 2009, Glazer et al. 2010). Among these proteins, only GAP65 has been suggested to be involved in the determination and stabilization of the amorphous polymorph.

How the biogenic ACC polymorph can be stabilized in time is still an open question that received recent attention. It was previously suggested that specialized macromolecules (acidic proteins, phosphoproteins, sulfated glycoproteins) or ions such as magnesium or phosphate could contribute to this stabilization (Aizenberg et al. 1996, 2002, Raz et al. 2000, 2003, Addadi et al. 2003, Luquet and Marin 2004, Marin and Luquet 2007, Shechter et al. 2008, Bentov et al. 2010). Very recently, Sato and co-workers (2011) and Akiva-Tal and co-workers (2011), by using cucticle and/or gastrolith from *Procambarus clarkii* and *Cherax quadricarinatus* as models, respectively, presented a new insight into induction and stabilization of ACC. By using solid state NMR spectroscopy, they demonstrated the presence of phosphorylated energy-rich intermediates of the glycolytic pathway and suggested their possible involvement in these processes. ACC particles would form first due to the interaction

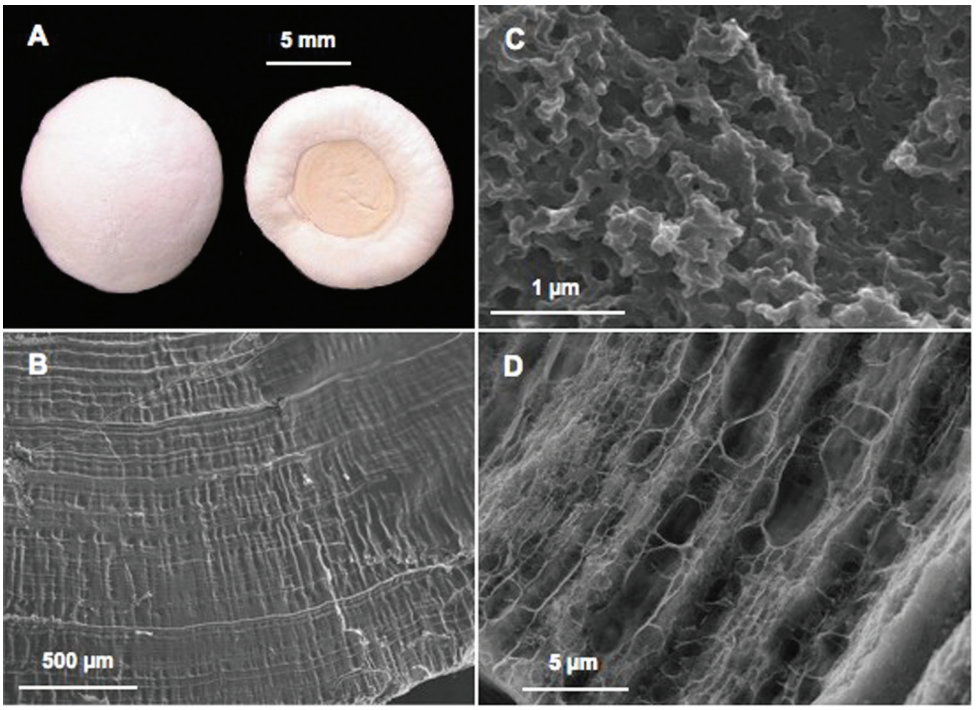

Figure 3. Calcium storage as gastroliths in decapods. **A** Pair of gastroliths from the crayfish *Cherax quadricarinatus* (Light Microscopy) **B** Internal striated structure visible on natural fracture after slight acetic acid decalcification (SEM) **C** Ultrastructure well visible after natural fracture and high magnification (SEM): the mineral is precipitated as nanospheres **D** Organic matrix network revealed after acetic acid decalcification (SEM).

of specialized matrix macromolecules bound to chitin in the $CaCO_3$ precipitation process. Then phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3PI) would act by binding to the surface of ACC, thus inhibiting the transformation of ACC into a crystalline polymorph (Sato et al. 2011). Citrate might be also involved in ACC stabilization by forming citrate-Ca^{2+}-P complexes (Akiva-Tal et al. 2011). Similarly, it is to notice that NMR analyses revealed that the amorphous mineral storage structures found in the hepatopancreas of the crab, *Ucides cordatus*, are phosphate-rich granules containing mainly orthophosphate, but also pyrophosphate and glucose-6-phosphate, considered as possibly involved in the stabilization of the amorphous state (Corrêa et al. 2009).

Conclusion

The formation of the majority of biominerals is under biological control. The knowledge of the physical and chemical features of the matrix components (proteins, polysaccharides, proteoglycans, lipids, low-molecular weight components...) is a prerequi-

site to understand, at the molecular level, how a biomineralization is elaborated, how the matrix molecules are involved in the nucleation and precipitation processes, how they influence the determinism of the polymorph obtained, and finally how a demineralizing process may occur.

In consideration of their ability to cyclically elaborate and resorb biomineral composites, crustaceans appear as convenient models for such prospects. They are not only able to synthesize a calcified exoskeleton but also calcium storage structures, which differ in their morphology and in their mineral composition. The calcium storage deposits are transient reservoirs of calcium ions that must be quickly mobilizable after ecdysis, and, for this reason, the storage structures are composed mainly of ACC. The cuticle reveals also a remarkable biocomposite because of the simultaneous presence of different polymorphs of $CaCO_3$ and ACP as well. Recent investigations suggest that if some general structural features may be common to all cuticles, it seems likely that the cuticle mineral composition, linked to the molecular content, not only could vary from one order of crustaceans to another but could also depend on the ecophysiology of each species.

To understand why the amorphous calcium carbonate state is stabilized in time whereas the same purely inorganic mineral is completely unstable, as well as how the switch of the transformation from the amorphous to crystalline phases occurs, is also of great interest in the biomaterial and nanotechnology fields (Han and Aizenberg 2008, Gower 2008, Tao et al. 2009). From studies on crustaceans models it was evidenced that magnesium and phosphate ions, proteinaceous macromolecules and low-molecular weight phosphorylated components of the organic matrix could be responsible for this stabilization. If some recent results appear meaningful in this sense, unfortunately there are still too few matrix molecules really well characterized so far in crustaceans, as in other phyla, to have a clear idea of the complete process of elaboration of a biomineralization. Nevertheless, it seems more and more evident that the stabilization of the amorphous state, similarly to other processes such as $CaCO_3$ nucleation and precipitation and the simultaneous presence of different polymorphs of $CaCO_3$ are multi-parameter phenomena, which result from the synergistic cooperation of several if not all the categories of matrix components above described.

Finally, by means of comparative studies performed in other calcifying phyla, crustaceans are useful to determine why and how calcification could have emerged on Earth. The sequence analysis of the matrix proteins and the genes encoding these proteins could lead to the understanding of the strategy used by evolution to built and select different mineralizing systems: convergence of different biological systems for a similar mineralizing function by exaptation of initially non-mineralizing molecules or evolution and adaptative divergence from an ancestral biomineral system still undeciphered?

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RESEARCH ARTICLE

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Widespread *Wolbachia* infection in terrestrial isopods and other crustaceans

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Abstract

Wolbachia bacteria are obligate intracellular alpha-Proteobacteria of arthropods and nematodes. Although widespread among isopod crustaceans, they have seldom been found in non-isopod crustacean species. Here, we report *Wolbachia* infection in fourteen new crustacean species. Our results extend the range of *Wolbachia* infections in terrestrial isopods and amphipods (class Malacostraca). We report the occurrence of two different *Wolbachia* strains in two host species (a terrestrial isopod and an amphipod). Moreover, the discovery of *Wolbachia* in the goose barnacle *Lepas anatifera* (subclass Thecostraca) establishes *Wolbachia* infection in class Maxillopoda. The new bacterial strains are closely related to B-supergroup *Wolbachia* strains previously reported from crustacean hosts. Our results suggest that *Wolbachia* infection may be much more widespread in crustaceans than previously thought. The presence of related *Wolbachia* strains in highly divergent crustacean hosts suggests that *Wolbachia* endosymbionts can naturally adapt to a wide range of crustacean hosts. Given the ability of isopod *Wolbachia* strains to induce feminization of genetic males or cytoplasmic incompatibility, we speculate that manipulation of crustacean-borne *Wolbachia* bacteria might represent potential tools for controlling crustacean species of commercial interest and crustacean or insect disease vectors.

Keywords

Wolbachia, endosymbiont, Crustacea, Maxillopoda, terrestrial isopod, distribution, adaptation

Introduction

Wolbachia pipientis (hereafter *Wolbachia*) bacteria are obligate intracellular alpha-Proteobacteria of arthropods and nematodes (O'Neill et al. 1997; Bourtzis and Miller 2003). These maternally-inherited bacteria are often referred to as reproductive parasites because they are able to manipulate the reproduction of their hosts to increase their own transmission, via mechanisms such as cytoplasmic incompatibility, male killing, thelytokous parthenogenesis and feminization of genetic males (O'Neill et al. 1997; Bourtzis and Miller 2003; Cordaux et al. 2011). In addition to vertical transmission, *Wolbachia* bacteria are occasionally transmitted horizontally (Werren et al. 1995; Vavre et al. 1999; Cordaux et al. 2001). These transmission patterns probably explain, at least partly, why *Wolbachia* bacteria are found in a highly diverse range of hosts.

Wolbachia bacteria are particularly frequent in arthropods. Hence, it has been estimated that 20–75% of insect species may be infected by *Wolbachia* (Werren and Windsor 2000; Hilgenboecker et al. 2008). These bacteria have also been reported in chelicerates such as mites (Breeuwer and Jacobs 1996), spiders (Cordaux et al. 2001) and scorpions (Baldo et al. 2007). In crustaceans, *Wolbachia* have long been known to infect the terrestrial isopod *Armadillidium vulgare* (Rousset et al. 1992) (order Isopoda, suborder Oniscidea; classification from Martin and Davis (2001)), in which *Wolbachia* induce functional feminization of genetic males (Rigaud et al. 1997; Cordaux et al. 2004; Bouchon et al. 2008; Cordaux et al. 2011). A systematic search in 80 crustacean species suggested that *Wolbachia* infection was restricted to isopods, with a prevalence of 46% in terrestrial isopod species (Bouchon et al. 1998). Based on additional screenings, this figure has recently been updated to 61% (Bouchon et al. 2008). The initial screening of 80 crustacean species also provided molecular evidence for *Wolbachia* infection in two other isopod suborders (Asellotta and Flabellifera) (Bouchon et al. 1998).

Despite multiple screenings of crustacean groups, *Wolbachia* bacteria have seldom been found in non-isopod crustacean species (Bouchon et al. 1998; Fitzsimmons and Innes 2005; Maniatsi et al. 2010). To date, only two amphipod and two non-marine ostracod crustacean species outside of the Isopoda order have been reported to be infected by *Wolbachia* (Cordaux et al. 2001; Baltanas et al. 2007). The extent to which this uneven *Wolbachia* distribution among crustacean species reflects host spectrum specificity or biased sampling remains to be clarified. In this study, we report *Wolbachia* infections in fourteen new crustacean species. Our results extend the range of *Wolbachia* infection within terrestrial isopods and amphipods (class Malacostraca). Moreover, the discovery of *Wolbachia* in the goose barnacle *Lepas anatifera* (subclass Thecostraca) establishes *Wolbachia* infection in class Maxillopoda. Molecular characterization indicates that the new strains are closely related to B-supergroup *Wolbachia* strains previously reported from crustacean hosts (Bouchon et al. 1998; Cordaux et al. 2001). The identification of closely related *Wolbachia* strains in highly divergent crustacean hosts suggests that this group of *Wolbachia* endosymbionts can naturally adapt to a wide range of crustacean hosts, not just isopods.

Materials and methods

Wild-caught individuals belonging to fourteen crustacean species were studied (Table 1). Seven species have been previously detected in a survey of *Wolbachia* infection of woodlice fauna in Tunisia (Ben Afia Hatira et al. 2007). For these species, *Wolbachia* prevalence (ranging from 40% to 100%) is available in the original publication. The seven remaining species were included as part of an ongoing effort in our laboratory to sample and test novel crustacean species for *Wolbachia* infection. For these species, one or two individuals were collected. Therefore, no information on prevalence is available for these species. Genomic DNA from single individuals was extracted as previously described (Bouchon et al. 1998). *Wolbachia* infection status of each individual was tested using a PCR assay based on the standard *wsp* marker. We used primers 81f and 691r (Braig et al. 1998) and previously described PCR conditions (Cordaux et al. 2001). Purified PCR products were directly sequenced in both directions, as previously described (Cordaux et al. 2001). The *wsp* sequences generated in this study were deposited in GenBank under accession numbers HE616815-HE616830 (Table 1).

Table 1. Novel crustacean species infected by *Wolbachia* bacteria reported in this study.

Class	Infraclass (I) or Order (O)	Species	Sampling location	GenBank accession number for *wsp*
Maxillopoda	Cirripedia (I)	*Lepas anatifera*	La Rochelle, France	HE616817
Malacostraca	Amphipoda (O)	*Talitrus saltator*	La Rochelle, France	HE616820 and HE616821
	Isopoda (O)	*Armadillidium granulatum*	Sidi Massaoud Khniss, Tunisia	HE616828
		Armadillidium pelagicum	Ras Jbel, Tunisia	HE616829
		Armadillidium sulcatum	Natural Reserve Mhibes, Tunisia	HE616827
		Cubaris murina	Baie Mahault, Guadeloupe, France	HE616815
		Hemilepistus reaumuri	Metbasta, Tunisia	HE616816
		Platyarthrus hoffmanseggbi	Liniers, France	HE616818
		Porcellio albinus	Kibili, Tunisia	HE616830
		Porcellio buddelundi	Skhira cliff, Tunisia	HE616823 and HE616824
		Porcellio lamellatus	Menzel Jmil, Tunisia	HE616825
		Porcellio variabilis	Jbel Ouest, Tunisia	HE616826
		Porcellionides cingendus	Archigny, France	HE616819
		Trachelipus rathkei	Cosne Cours sur Loire, France	HE616822

Sequences were aligned using ClustalW as implemented in the software BioEdit ver. 7.0 (Hall 1999), followed by manual adjustments. Representative sequences from the B supergroup of *Wolbachia* diversity were included for comparison and two of the A supergroup as an outgroup, as previously described (Cordaux et al. 2001; Cordaux et al. 2004). There was a total of 618 positions in the dataset. Hypervariable regions were deleted because they could not be aligned with confidence. The resulting alignment included 512 positions of which 185 were considered informative by parsimony criteria. Recombination analyses were performed using Recombination Detection Program (RDP) v3.41 (Martin et al. 2010). Parameters were set as follows: sequences were considered linear, the highest acceptable P value cutoff was 0.01, a Bonferroni correction was applied, consensus daughter sequences were found, gaps were included, different window sizes of variable sites were tested (10, 20, and 30 VI), and 1,000 permutations were performed. Phylogenetic analyses were conducted using the Minimum Evolution (ME) and Neighbor-Joining (NJ) methods, as implemented in MEGA ver 4.0 (Tamura et al. 2007). Evolutionary distances were computed using the Kimura 2-parameter substitution model. The ME tree was searched using the close-neighbor-interchange algorithm at a search level of 1 and the NJ algorithm was used to generate the initial tree. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option). Bootstrap analyses were carried out with 1000 replicates.

Results and discussion

Our findings extend the range of *Wolbachia* infections among crustacean hosts to twelve additional terrestrial isopod species, one amphipod species and one cirriped species (class Maxillopoda) (Table 1). We performed a molecular characterization of the *Wolbachia* strains based on the *wsp* gene, a *Wolbachia*-specific genetic marker (Braig et al. 1998) which has been shown to be highly informative for the analysis of crustacean *Wolbachia* strains (Cordaux et al. 2001). In total, we identified sixteen different *Wolbachia* strains: two strains in the amphipod *Talitrus saltator* and the terrestrial isopod *Porcellio buddelundi*, and one strain in each of the other crustacean host species. This is the third report of multiple *Wolbachia* strains being harbored by a single terrestrial isopod host species and the first case reported in amphipods. Indeed three different *Wolbachia* strains have previously been identified in *A. vulgare* (Cordaux et al. 2004; Verne et al. 2007) and *Porcellionides pruinosus* (Michel-Salzat et al. 2001). As in *A. vulgare* and *P. pruinosus*, the *Wolbachia* strains found in *T. saltator* and *P. buddelundi* were identified in different individuals, and no multiple infection of single individuals have been reported so far in crustaceans, in contrast to what has been widely documented in insects (Vautrin et al. 2007).

Querying GenBank with the sixteen *wsp* sequences through BLASTN searches resulted in best matches to other *Wolbachia* strains with 98.0–100% nucleotide similarity. This analysis revealed that all novel *Wolbachia* strains belong to the B supergroup of *Wolbachia* diversity, as do all isopod and amphipod *Wolbachia* strains characterized

to date (Rousset et al. 1992; Bouchon et al. 1998; Cordaux et al. 2001; Michel-Salzat et al. 2001; Cordaux et al. 2004).

To further characterize the novel crustacean *Wolbachia* strains, we performed a phylogenetic analysis of B supergroup *Wolbachia* strains. To facilitate comparisons with previous analyses of crustacean *Wolbachia* strains, we added the sixteen strains reported in this study to the set of representative B supergroup strains previously used in Cordaux et al. (2001). No significant support for recombination was detected with RDP and the topologies of the ME and NJ trees (see Materials and Methods) reconstructed in this study were highly similar, as they were to the phylogenetic inferences reported previously (Cordaux et al. 2001). We also emphasize that, as far as crustacean *Wolbachia* strains are concerned, we have shown previously that a *wsp*-based phylogeny yielded essentially the same relationships between taxa as phylogenies based on other *Wolbachia* genes such as *16S rRNA*, *ftsZ* and *GroE* (Bouchon et al. 1998; Cordaux et al. 2001; Wiwatanaratanabutr et al. 2009). Because our updated phylogeny of crustacean *Wolbachia* strains is in agreement with phylogenetic results obtained in previous studies using various genes, we are reasonably confident that the phylogenetic analysis we present in the manuscript is reliable. Figure 1 shows the tree inferred from the ME analysis. All but one crustacean *Wolbachia* strains clustered together in one of the two groups *Oni* and *Rug*, previously shown to encompass isopod and amphipod *Wolbachia* strains (Bouchon et al. 1998; Cordaux et al. 2001; Wiwatanaratanabutr et al. 2009). Our extended dataset shows a newly emerging trend in the diversity of crustacean *Wolbachia* strains, in that the *Oni* group mostly contains strains isolated from terrestrial isopods, whereas the *Rug* group mostly contains strains isolated from non terrestrial isopod crustaceans. We speculate that this might reflect two major ancestral *Wolbachia* acquisitions in crustaceans, in terrestrial and aquatic environments. Ecosystems in the transition zones from land to sea, as estuaries and wetlands, are highly productive and therefore attract a multitude of wildlife. Moreover these ecosystems are in a continuous state of change. The finding of closely related symbionts in the *Rug* group suggesting recent symbiont dispersal across divergent hosts sharing the same habitats suggests that horizontal transfers of *Wolbachia* could be facilitated in such ecosystems.

The only exception to the clustering of crustacean *Wolbachia* strains in the *Oni* or *Rug* groups is the *Wolbachia* strain isolated from the terrestrial isopod *Cubaris murina* (Fig. 1). Indeed, the latter strain falls within the *Pip* group, closely related to *Wolbachia* strains from mosquitoes and drosophila. This result suggests the possibility of a *Wolbachia* horizontal transfer between isopod and insect hosts, as previously proposed to explain the similarity between strains of the *Rug* group with other insect *Wolbachia* strains (Bouchon et al. 1998; Cordaux et al. 2001). In any event, it is noteworthy that *C. murina* is a terrestrial isopod with a pan-tropical distribution (Schmalfuss 2003). Interestingly, a similar result has been obtained by Wiwatanaratanabutr et al. (2009) who showed that the *Wolbachia* strain isolated from an endemic isopod sampled in Thailand also belongs to the *Pip* group. This is in contrast to most terrestrial isopods screened so far for *Wolbachia* infection, which were generally sampled in temperate regions (Bouchon et al. 1998; Bouchon et al. 2008). This suggests that screenings of

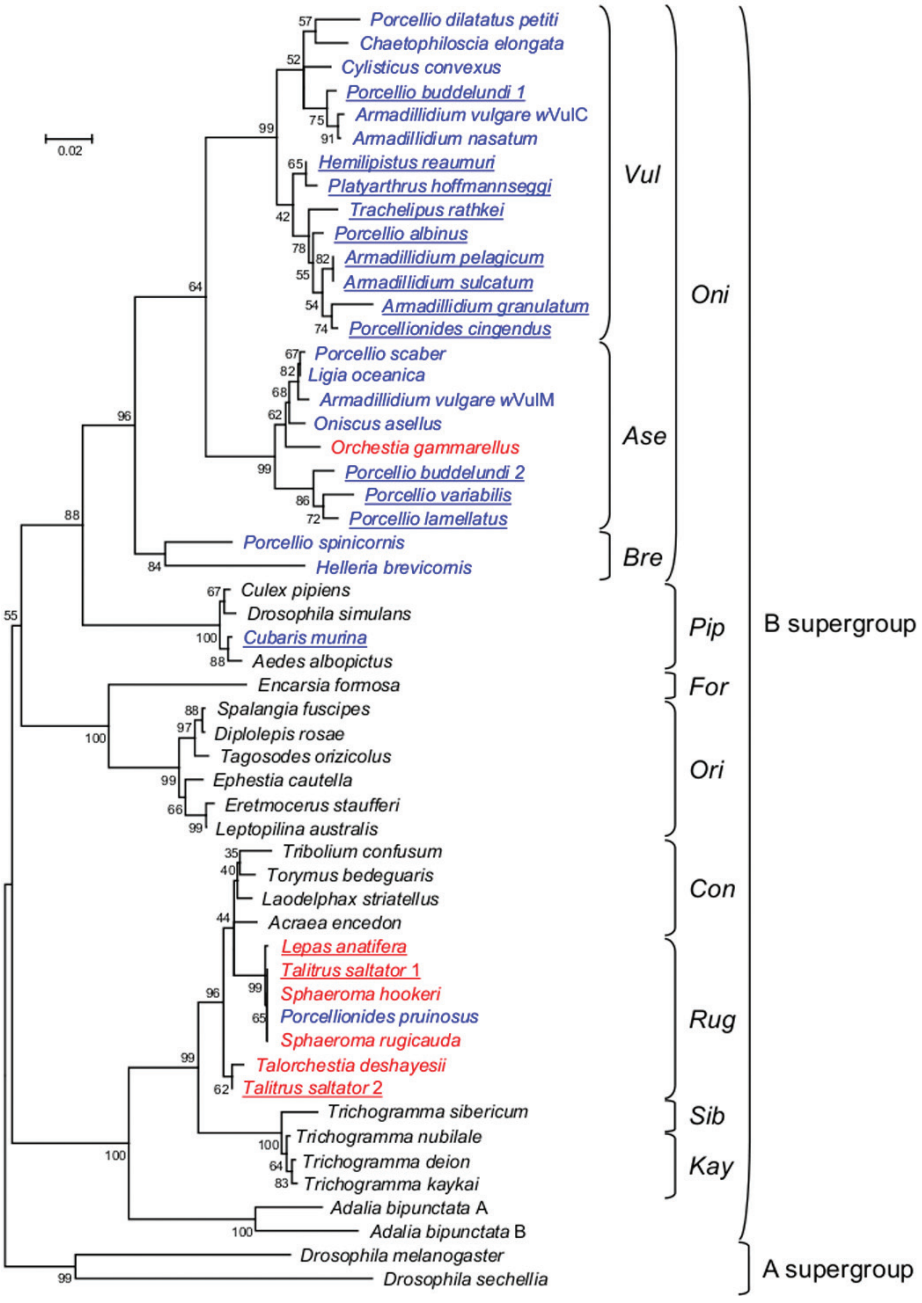

Figure 1. Phylogenetic tree of B-supergroup *Wolbachia* strains based on *wsp* sequences, using Minimum Evolution analysis. The tree is rooted with two A-supergroup *Wolbachia* strains. Bootstrap values inferred from 1000 replicates are shown as percentages. Strains are identified by the host species from which they were isolated. *Wolbachia* strains from terrestrial isopods and non terrestrial isopod crustaceans are shown in blue and red, respectively. New crustacean *Wolbachia* infections reported in this study are underlined. *Wolbachia* strains from insects are shown in black. Names assigned to groups of *Wolbachia* strains are shown on the right, following Cordaux et al. (2001).

terrestrial isopods and other crustaceans in regions that have not been extensively studied so far may have the potential to uncover a tremendous and unexpected *Wolbachia* diversity that we are only beginning to realize.

An important result of this study is the discovery of *Wolbachia* in the goose barnacle *Lepas anatifera*. This is the first report of *Wolbachia* infection in the class Maxillopoda, a major crustacean group comprising ~15,000 species, that is, more than one quarter of all described crustacean species (Martin and Davis 2001). Interestingly, the Maxillopoda *Wolbachia* strain is closely related to other crustacean *Wolbachia* strains in the *Rug* group, being 99.8% similar to the *Wolbachia* strains from the terrestrial isopod *Porcellionides pruinosus*, the intertidal isopods *Sphaeroma rugicauda* and *S. hookeri* and the amphipod *T. saltator*, based on the *wsp* marker (Fig. 1). This result thus indicates that highly divergent crustacean hosts can harbour highly similar *Wolbachia* endosymbionts. By implication, our results suggest that this group of *Wolbachia* endosymbionts can naturally adapt to a wide range of crustacean hosts. Given the demonstrated ability of terrestrial isopod *Wolbachia* strains to induce feminization of genetic males or cytoplasmic incompatibility between infected males and uninfected females (Legrand and Juchault 1986; Rigaud et al. 1997; Bouchon et al. 1998; Moret et al. 2001; Cordaux et al. 2004; Bouchon et al. 2008; Cordaux et al. 2011), we speculate that manipulation of crustacean-borne *Wolbachia* bacteria might represent potential tools for controlling crustacean species of commercial interest and crustacean or insect disease vectors. For example, it has been shown that freshwater crustaceans could be used as predators to control immature forms of the mosquito *Aedes aegypti*, the vector of the Dengue fever, one of the major infectious diseases in several tropical and subtropical countries in Asia, Africa, and The Americas (Kosiyachinda et al. 2003). The finding of *Wolbachia* infection in a wide range of crustacean species, including freshwater crustaceans, may open new opportunities in the biological control of insect disease vectors via a *Wolbachia*-based strategy. This might allow researchers to manipulate the population dynamics of crustacean predators of insects, to enhance their efficacy as biological control agents.

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RESEARCH ARTICLE

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Aggregation in woodlice: social interaction and density effects

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Abstract

Terrestrial isopods are known to be sensitive to humidity, brightness or temperature. Until now, aggregation was assumed to depend on these sensitivities as a result of individual preferences. In this paper, we show that the social component is also important in the isopod aggregation phenomenon. In experimental arenas with two identical shelters up to nearly 90% of woodlice aggregated under shelters. This aggregation was quick as in 10 minutes most of the animals aggregated, irrespective of their density. Nonetheless, 10–15% of the animals walked around the arena, rarely forming very small and short-lasting aggregates outside shelters. Woodlice aggregated preferably under one of the shelters in 77% of experiments. Indeed, almost 80% of the animals out of 40, 60 or 80 animals in the arena aggregated under one shelter. In arenas with 100 individuals the aggregations were proportionally smaller (70%). Our results revealed that 70 animals was a maximum number of woodlice in an aggregate. We concluded that the location of aggregates is strongly governed by individual preferences but the dynamics of aggregation and collective choice are controlled by social interaction between congeners. The tested densities of the animals in the arena did not impact the aggregation patterns.

Keywords

Woodlouse, aggregation, social interaction, density, dynamics

Introduction

Woodlice are mainly detritivorous organisms feeding on leaf litter, decayed wood, fungi, and bacteria. They are one of the most important groups of organisms driving the

dynamics of soil (Hassall et al. 1987, Zimmer et al. 2002). In European woodlands the density of woodlice is very variable and can reach 800 individuals/m²; however, in some calcareous grasslands their density can reach 3000 individuals/m² (Gongalsky et al. 2005, Paoletti and Hassall 1999). These measures given per m² do not really reflect densities observed at the scale of micro-habitat used by woodlice. Indeed, it is frequently observed that there is a strong variation of density between different micro-habitats in similar environments with ranges from less than 10 individuals to more than 60 congeners (Davis and Sutton 1977, Gongalsky et al. 2005, Paris 1963). The observation of such variation can be explained by the individual preferences of woodlouse in heterogeneous environments with different qualities of micro-habitats. Individual preferences of woodlouse are well known and have been strongly studied in the past (Cloudsley-Thompson and Constantinou 1987, Sutton 1972, Sutton and Holdich 1984). However, recent studies have shown the importance of social interactions in woodlice aggregation (Devigne et al. 2011). Hence, aggregation patterns observed cannot be explained only with individual preferences but they result from synergy and competition between such preferences and the social interaction between individuals. This new approach in the understanding of woodlice aggregation will permit us to better study how the individuals could be distributed in an environment. In consequence, woodlice distribution will depend on the micro-habitats available but also will depend on the density of congeners, the social interactions being density dependent. This paper aims to give new insights about the speed of the aggregation dynamics and collective choice made by groups of woodlice in standardized experimental conditions. This paper specifically deals with the impact of density of congeners on the characteristics of aggregation process.

Methods

Rearing conditions

The rough woodlouse, *Porcellio scaber* Latreille, 1804 is a widely distributed terrestrial isopod well known to form aggregates. Individuals were collected in the gardens of Lille Catholic University (Northern France). They were reared in terraria (410x240x225mm) on a plaster layer regularly moistened (H°=75 ±10%). They were fed with litter of maple, beech and oak leaves. Room temperature (as well as the experimental set-ups) was kept at 23 ±2°C. Photoperiod was 14:10 (L:D).

Experimental set-up

The experimental set-up consisted of a circular arena (diameter193mm) with two dark shelters (Fig. 1). The experimental set-up was placed on a white sheet of paper which was changed between each experiment.

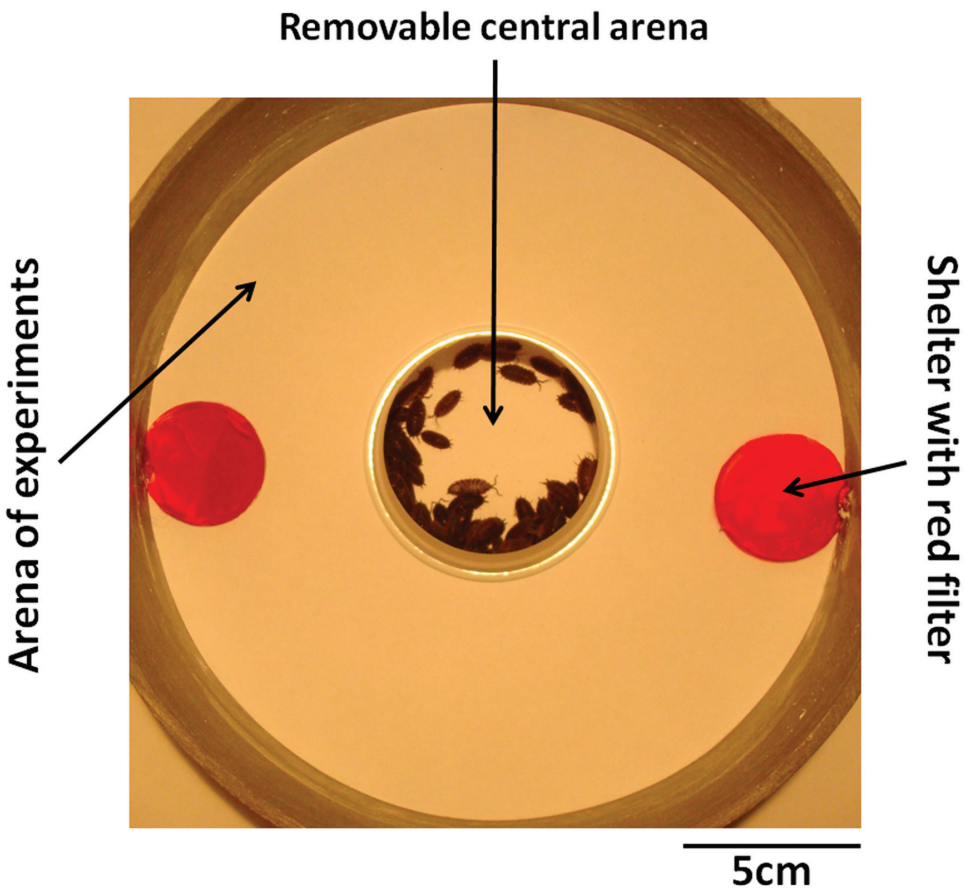

Figure 1. Experimental set-up.

Shelters consisted of a small glass plate (diameter 35mm placed at 5mm of soil). Darkness in shelters was achieved by adding to glass plates, two layers of red ROSCO® filters (ref. Roscolux #19 Fire – this filter changed the spectrum of light by transmitted to nearly only red energy). The set-up was lit with 156 lux and the brightness under both shelters was only 41 lux. Both shelters in the arena were strictly identical in size, darkness and contact surface with the edge of the arena. No bias between the number of woodlice observed under the left and the right shelter could be found by analyzing the whole data (Wilcoxon's test, p=0.263, N=87).

Before the experiments, woodlice were placed in groups of 40 (N=29), 60 (N=20), 80 (N=20) or 100 (N=19) individuals in the centre of the experimental arena in a small removable central arena (diameter 65mm – Fig. 1). When the animals were calm (after about 5 minutes) the small central arena was removed and the aggregation dynamic was video-recorded during 45 minutes (thanks to a Sony camera CCD firewire - DMK 31BF03). Hence, densities used, in these experiments, ranged from 1325 to 3315 individuals/m².

Data analysis

In order to determine whether woodlice selected one shelter preferentially, binomial tests were carried out with H_0 assuming an equal distribution of woodlice between both shelters. After this binomial test, it is possible to define the "*winning*" shelter as being the shelter with the higher number of woodlice at the end of the experiment and the "*losing*" shelter as the other one (for the method, see Sempo et al. 2009).

X^2 test was used to compare the proportion of experiments with choice of one shelter according to density.

Since our data did not meet conditions for parametric tests, comparisons of results obtained with different densities were carried out with a Kruskal-Wallis test followed, if necessary, by a Dunn's test.

GraphPad software InStat 3 was used to carry out the statistical tests.

Results

First of all, only one of all 88 replicates did not show any aggregation during the 45 minutes of observation. Hence, this replicate was not considered. In all the other replicates, regardless of the density, nearly 90% of woodlice were observed to aggregate under shelters after 45 minutes (Table 1). No experiments showed a large aggregation outside shelters at the end of the 45 minutes. However, some woodlice (less than 15%) generally still walked around in the arena (Table 1) rarely forming very small aggregates (only two observations in the 87 experiments carried out).

Experiments showed that groups of woodlice generally selected one of both shelters (Fig. 2). Indeed whatever the density condition, more than 77% of all replicates (regardless of isopod density) showed a clear selection of only one shelter (Fig. 2. c^2 test, c^2=0.17, p=0.98 – no difference between density conditions).

In order to understand the aggregation dynamics, separate analyses of replicates with a clear choice of one single shelter (77%, N=87) and replicates where isopods reparted almost equally among the two shelters, i.e. no selection of one shelter (23%) were necessary. However, the number of replicates without choice was low and were evenly distributed among the four densities tested (Fig. 2). Hence, in the remaining part of this paper only replicates with choice will be described and discussed.

Table 1. Proportion of aggregated woodlice and proportion of woodlice under shelters or outside shelters at the end of experiments.

	Proportion of aggregated woodlice (%)	**Proportion of woodlice under shelters (%)**	**Proportion of woodlice outside shelters (%)**	**N=**
40 woodlice	88.2 (± 7.1)	87.1	12.9	29
60 woodlice	87.4 (± 7.5)	87.4	12.6	20
80 woodlice	88.4 (± 7.0)	88.4	11.6	20
100 woodlice	89.1 (± 5.6)	89.1	10.9	18

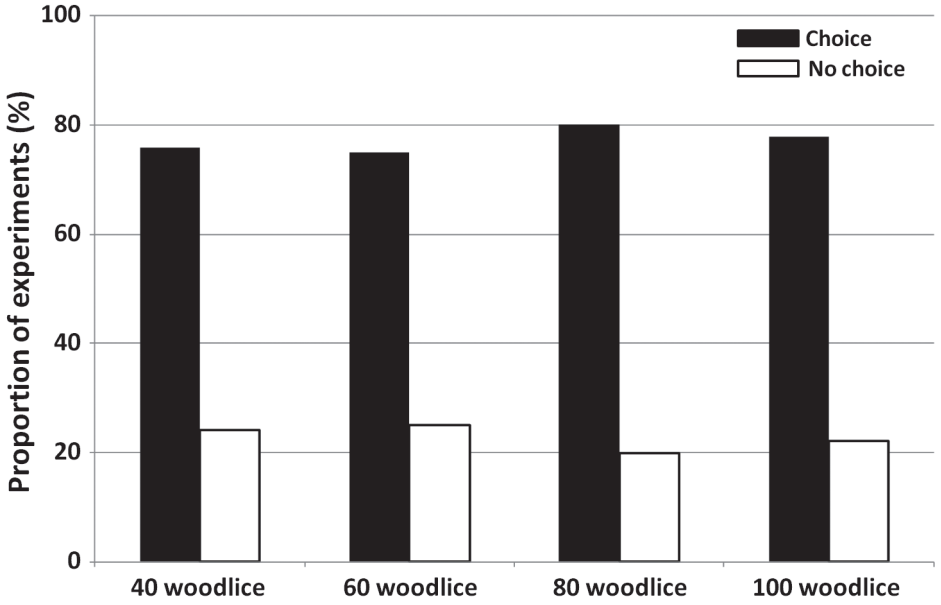

Figure 2. Choice of one shelter. Proportion of choice of a shelter at the end of the experiments as a function of woodlice density.

Woodlice showed a strong thigmotactic behaviour; just after their release, woodlice walked in the arena, generally near the edge and they quickly entered under both shelters (Figure 3). The number of woodlice increased simultaneously under both shelters but, most woodlice quickly concentrated under one shelter (Figure 3). In less than 3 minutes on average, one aggregate was larger under one of the shelters and it remained larger during the experiments. This result was observed at any density condition tested (time of selection was 2.18±1.5, 2.06±2.28, 1.69±1 and 2.14±1.6 minutes for treatments with 40, 60, 80 and 100 woodlice set-ups, respectively; Kruskal-Wallis' test p=0.42). Whatever the density, the proportion of woodlice under the "winning" shelter quickly increased to more than 50% of woodlice in less than 10 minutes for each treatment (Fig. 3). After 10 minutes, the proportion of aggregated woodlice under the "winning" shelter slightly increased to stabilize at nearly 80% of woodlice for 40, 60 and 80 woodlice experiments and around 70% with 100 woodlice set-up at the end of the experiments (Fig. 3). However, there were significantly more woodlice under the "winning" shelter when there are more woodlice in the set-up except when the number of woodlice is higher than 80 (Fig. 4 – Kruskal-Wallis test). The proportion of aggregated woodlice under the "losing" shelter was around 7–10% of woodlice in 40, 60 and 80 woodlice set-ups (Fig. 3 – average of 10.7±10.7, 7.1±10.8 and 9.1±10.5% for 40, 60 and 80 woodlice set-ups, respectively). This proportion reached 20.4±12.7% in set-ups with 100 woodlice (Fig. 3). The individuals which were not found under the shelters were observed walking in the arena. Whatever the experiments, these walking woodlice generally consisted in 10–15% of population introduced.

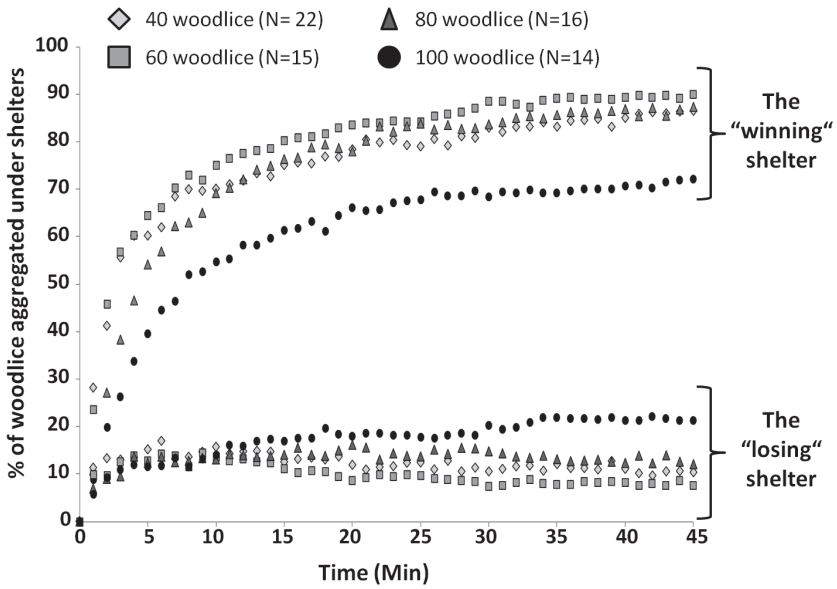

Figure 3. Dynamics of aggregation under shelters. Average proportion of woodlice aggregated under the "winning" and the "losing" shelter for experiments showing a clear choice of one of both shelters (Binomial test, difference from an equal distribution of woodlice between shelters).

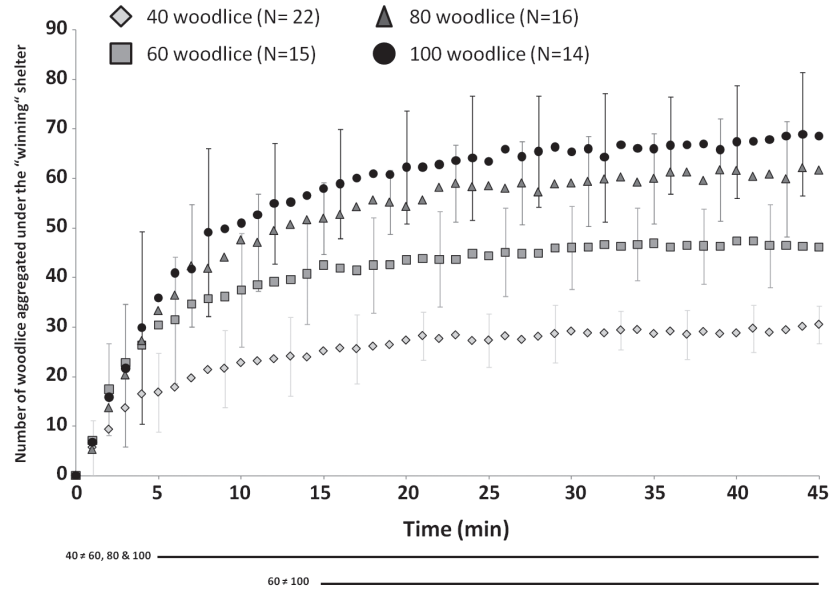

Figure 4. Dynamics of aggregation under the "winning" shelter. Evolution of the average number of woodlice under the winning shelter as a function of time for the four densities tested and for experiments showing a clear choice of one of both shelters. Standard deviations are presented for each 4 minutes. Horizontal lines below the graph indicated the statistical differences between densities; these differences were pointed out by a Kruskal-Wallis followed by a Dunn's tests for each minute of the experiments.

Discussion

The densities used in this study do not impact the aggregation process. Indeed, no differences were observed between density conditions in the dynamics of aggregation, the collective choices and the rates of selection of only one shelter. Aggregation in woodlice is very frequent (Allee 1931; Friedlander 1965). Only one of all 88 replicates did not show any aggregation during 45 minutes. Woodlice aggregation always occurred under shelters, i.e. under reduced light conditions. Small aggregations observed outside shelters were not stable. Hence, individual preferences ruled the location of aggregates and our results confirmed that populations of woodlice were able to select one shelter when two identical shelters were available (Devigne et al. 2011). This study shows, for the first time, that this collective choice is not impacted by woodlice density. With a very high proportion of aggregated woodlice, only 10–15% of individuals were observed walking on the arena at the end of experiments, regardless of the density treatment. Although most of the characteristics of the aggregation did not vary with the density, some particularities deserve a discussion which will point out the complexity of mechanisms which come into play during this phenomenon which have been often tackled but are still not well understood. This discussion will also give rise to new issues for future investigations.

In more than 77% of experiments, a choice of one shelter was made by groups of woodlice. In such experimental conditions, these selections can only be explained by the social interactions between congeners (Camazine et al. 2001, Jeanson and Deneubourg 2007, 2009). However, individual preferences are important since even with higher densities, woodlice never aggregated outside the shelters. These results moderate our first observations which showed that social interactions could outweigh individual preferences in the collective decision making by leading the groups toward suboptimal choices (Devigne et al. 2011). In this respect, observation of systematic aggregation under shelters could be explained by the high density in our experiments. Indeed, the increase of density can enhance the efficiency of collective choice and hence to decrease the frequency of suboptimal choices (Canonge et al. 2011, Sempo et al. 2009). Moreover the expected increase of the selection rate of only one shelter due to higher density was not observed: the same proportion of experiments showed a selection of one shelter whatever the tested densities. At the higher density, the main aggregation reached a plateau (around 70 woodlice in our conditions) and most of the other woodlice were found under the second shelter. Hence the lack of increase of selection rate with density could result from a saturation of the selected shelters (see below).

Concurrently to the absence of an enhanced selection rate, our results did not show the expected acceleration of aggregation dynamics, in conjunction with higher density. Indeed, this phenomenon being driven, in part, by social interactions between congeners, aggregation in a preferred shelter should happen faster at higher densities. In our experiences, whatever the number of woodlice, the aggregation was very quick (in less than 10 minutes most of woodlice are aggregated) and did not differ between densities. Possibly, the aggregation process was already very quick even at our low-

est density used (1325 individuals/m² corresponding to 40 individuals) so that the phenomenon could not happen any faster. The density used in these experiments corresponded to the high values observed in nature (Gongalsky et al. 2005, Paoletti and Hassall 1999) but lower densities are often observed in the field. More investigations with lower densities and also at other spatial scales –density-dependence relations can vary according the spatial scale (Courchamp et al. 2008)– should allow us to identify the relative part of individual preferences and social interaction in the aggregation dynamics and better understand the diversity of aggregation patterns.

The number of woodlice aggregated under the "winning" shelter increased with the number of woodlice within the setup. However, from a number of 80 woodlice in the set-up, the number of woodlice under the "winning" shelter reaches a plateau around 70 woodlice (no difference was found between 80 and 100 woodlice set-ups). This result firstly suggests a saturation of shelters at 70 woodlice. This may result from the shelter carrying capacity. Nevertheless, a stable aggregation under a shelter whatever the density, often extended beyond the edge of that shelter. As a consequence, some woodlice belonging to the aggregation were not in the darker area. Keeping in mind that at the 100 woodlice condition, a second stable aggregate grows under the losing shelter, two "functional" hypotheses, deserving new investigations, can explain this maximal number of woodlice in an aggregate. Firstly, it is possible that competition in the aggregate increases with the number of woodlice and beyond 70 woodlice, it could be better for a woodlouse to join a smaller aggregate (Brereton 1956, Lefebvre 2002, Paris 1963, Thiel 2011). Secondly, the benefits of aggregation concerning the reduction of water loss could decrease at large cluster size (Allee 1926; Cloudsley-Thompson and Constantinou 1987, Edney 1951, Gunn 1937, Warburg 1964). Indeed, from a size of around 60–70 aggregated individuals, the woodlouse did not reduce their water losses (Kuenen and Noteboom 1963, Broly et al. In prep). Therefore it could try to join another smaller aggregate but with the opportunity to be under a shelter. Besides, aggregates with more than 70 woodlice were sometimes observed. However, the activity of woodlice was higher and these aggregates are transitory and hence unstable.

These results were in accordance with the existence of aggregation pheromone coming from faeces suggested by the past (Kuenen and Noteboom 1963, Takeda 1984). However the speed of the aggregation (in 10min) questions about the implication of such potential pheromone coming from faeces. Indeed during the experiments, woodlice produce a small amount of faeces. Other pheromones released by individuals could potentially be involved in the aggregation process. However specific experiments dealing with the implication of pheromone in aggregation process currently occur to decipher the part and the role of such signal during the aggregation in *Porcellio scaber*.

In the field, in woodlice and most of the organisms, the local population densities depend on characteristics of their environment (litter, Zimmer and Topp 1997; temperature or humidity, Zimmer and Brauckmann 1997). Moreover, in the species social interactions can impact the spatial distribution by promoting aggregated patterns. Better knowledge about aggregation processes and measures of density at small scales would allow us to understand their spatial distribution in nature (Detsis 2009,

Grear and Schmitz 2005, Kao 1984, Tremblay and Gries 2006). Our results showed that location of aggregates is strongly governed by individual preferences and that the dynamics of aggregation and collective choice are controlled by social interaction between congeners. Nevertheless, densities did not impact the aggregation patterns. That could seem surprising because when the number of woodlice increases, potential interactions increase too and dynamics should be modified. Maybe the densities chosen in our experiments were too high to observe any change. However, our results showed that maximum number of woodlice in a cluster is reached in high density conditions. If our results showed a maximum number around 70 woodlice in a cluster, besides shelter size this value certainly depends on environmental conditions (e.g. humidity) or physiological state of woodlice inside aggregates.

Moreover, a complete understanding of the woodlice aggregation and its characteristics needs a theoretical approach of the costs and benefits of the aggregation in order to evaluate the differences for woodlice between optimal and stable sizes of clusters (Krause and Ruxton 2002, Sibly 1983, Thiel 2011).

Social interactions in woodlice and different environmental parameters (such as maximum carrying capacity of shelters or maximum size of aggregates) are important to understand the distribution of woodlice in the environment. In natural conditions, a local peak of population (in case of binary choice, the population is higher on one side) may result from the coupling between the response to the environmental heterogeneities and the social interaction. Moreover, even if more investigations are necessary to decipher the mechanisms explaining, the velocity of gathering in aggregates, the maximum size of clusters and the social signals used we suggest that similar observations could be made now in field.

Since woodlice are often used as bioindicators for pollution, the explanation of the collective decision making and patterns of aggregation of woodlice population could inform us about quality of environment (Godet et al. 2011, Loureiro et al. 2006, Zidar et al. 2004, 2005) and could improve experimental tests used to assess soil contamination (Kaschl et al. 2002, Loureiro et al. 2005, Zidar et al. 2002). The social interaction, amplifying the individual response, could explain the disagreement between the response of isolated individuals and the response of a group as suggested by Loureiro et al (2005). If group size effect on the survival rate are well-known (Allee effect, Brockett and Hassall 2005), future studies on choice, preference and avoidance could take account these social effects and the size of the tested population (at least isolated individuals vs groups) that could affect the experimental results and the conclusions.

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RESEARCH ARTICLE

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The effect of external marking on the behaviour of the common pill woodlouse *Armadillidium vulgare*

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Abstract

Zoologists distinguish individual animals using marking techniques. Generally they test the potential influence of marking on survival only; the influence on behaviour is usually neglected. We evaluated the influence of two external marking techniques (nail polish and queen-bee marker) on the behaviour of common pill woodlouse, *Armadillidium vulgare*. The behaviour was examined from two points of view: (1) activity during 24 hours and (2) specific expressions of behaviour (exploring, feeding, resting and hiding) over a 24 hour period. We compared behaviour among woodlice marked with nail polish and queen-bee marker with the unmarked control group during a nine-day experiment. Although we did not find any influence of marking on survival, there was an evident influence on behaviour in most cases. Generally, in the groups of marked individuals of *A. vulgare* there were large differences observed against the control group in the overall activity. Activity of marked individuals was significantly reduced and they preferred hiding. The influence of polish and marker on the overall frequencies of behavioural categories was evident, mainly in feeding, resting and hiding. The influence on the frequency of exploring was significant in the polish marked group only.

Keywords

Diurnal activity, external marking, influence on behaviour, daily pattern, Isopoda, Oniscidea

Introduction

From time to time zoologists need to distinguish individuals of model species. Individual identification is important in ecological studies (e.g. migration or population size) as well as in ethological studies (e.g. home range or social hierarchy). Researchers are able to use indi-

vidual phenotypic/genotypic differences to identify individuals of some vertebrate species (cf McGregor and Peake 1998) but this approach is a waste of time in studies of animals with short life spans such as many invertebrates. Several methods of marking invertebrate animals have been developed. Internal marking methods used in invertebrates are based generally on colouring and are suitable mainly for unpigmented animals (e.g. termites, tiny spiders or woodlice). Other internal marking methods are based on using isotopes (radioactive or stable ones) but they are limited mainly to population studies (Southwood and Henderson 2000). Paris (1965) also used this method in a study of common pill woodlouse dispersal. More frequently external marking methods are used in studies of invertebrates. They are especially used for marking of adult insects. Beside scarification (e.g. deformations of beetle elythrae by rasper or laser) and tagging (labels with code on locusts, molluscs etc.), painting is one of the most popular methods of external marking. Painting of woodlice has been used during laboratory and field studies of their life history (Lawlor 1976, Madhavan and Shribs 1981), shelter fidelity (Brereton 1957, den Boer 1961) and vagility (Paris and Pitelka 1962). A typical substance used for marking woodlice has been "enamel", substituted by nail polish in the study of Madhavan and Shribs (1981).

Acceptable methods for animal marking should not affect survival (such as increasing probability of predation or infection, or causing intoxication) or behaviour of marked individuals. The potential influence of marking on survival of marked animals is often evaluated but the influence on behaviour is generally neglected (cf Gallepp and Hasler 1975). Hence we decided to investigate if external marking could influence the behaviour of the common pill woodlouse, *Armadillidium vulgare* (Latreille, 1804) using two external marking methods: nail polish and queen-bee marker. Our study also aimed to investigate the potential influence of marking on survival.

Materials and methods

Biological material and marking process

Common pill woodlice, *Armadillidium vulgare,* were hand-collected in Olomouc City (Czech Republic). Collected animals of similar size were sorted out and reared in plastic boxes under room conditions (approx. temperature 21°C, almost 100% air humidity in boxes, natural summer photoperiod, sufficient raw potato food, stones as shelters). Three groups of 40 individuals were chosen for the experiment. Both first and second group were marked, the third group was left unmarked and served as a control.

The two external markings selected for the experiment were nail polish (60 seconds RIMMEL London™) and queen-bee marker (Uni Paint Marker™). The fast-drying nail polish was selected to reduce the probability of bonding tergites or sticking of an individual to the surface. Animals were picked up gently with two fingers, marked quickly with a small dot of marking agent on the first pereion segment and placed back into the box. The control group was also manipulated (i.e. picked up and placed in a box, but without marking agent).

Experimental design

The experiment was performed during August 2009. Individuals from polish-marked, marker-marked and control groups were placed in groups of 4 to a box (box size 20×20×10 cm with 0.5 cm layer of plaster of Paris). A box with 4 randomly chosen individuals from one group was considered as one sample. Each box was divided into thirds: the first third contained 3 shelters made from dark but see-through red plastic, the second third contained 40 g of fine soil and the last third contained 3 pieces of potatoes as food. After sunset a red coated flashlight was used to minimize the disturbance of individuals. There were 10 repetitions of each treatment, i.e. 30 boxes altogether. After the marking process, individuals were left to acclimatize in the experimental boxes for 2 days. Observations were performed for 24 hours on the 3rd, 6th and 9th day after marking. The actual behaviour of each individual was recorded once each hour with the naked eye. Active behavioural categories were recorded as: *exploring* (walking), *monitoring* (staying with moving antennae), *cleaning* (clearing of antennae or legs), *interacting* (contact with another individual outside soil or shelter) or *feeding* (feeding on potato, excrements or soil, drinking or defecation). Inactive behavioural categories were recorded as *hiding* (inactivity in soil or in shelter) or *resting* (inactivity on surface).

Statistical analysis

The effect of marking on survival of woodlice was tested by comparing the number of dead individuals from groups using a Fisher's Exact Test. To study behavioural responses to treatment, each behavioural category was defined as proportion of individuals from the group of 4 individuals in the same box exhibiting this particular type of behaviour. The four commonest (see below) categories of behaviour were evaluated, i.e. *feeding*, *exploring*, *resting* and *hiding*. Because time of day clearly acts as a strong confounding variable with a non-linear effect on behaviour of animals during the day, we decided to include this variable in the model structure. We analysed the effect of treatment (3 levels: control, marker and polish) on proportions of the exhibited type of behaviour by fitting generalized additive models (GAMs) which are capable of accounting for nonlinearity imposed by time of day, thereby leaving residuals for category testing. We set binomial error distribution and logit link function to model the effect of both predictors. We used package *mgcv* in program R (Wood 2006) which is exceptional by solving the smoothing parameter estimation problem as part of the estimation procedure. This procedure also provides approximate p-values for the null hypotheses that each term is zero. We modelled behavioural activities for the 3rd, 6th and 9th day separately. The smoothing term for time of day was always significant justifying the presence of this variable in the model. The effect of marking on *activity* (we analysed the main active categories, feeding and exploring, jointly) was visualized in program Oriana for Windows and also analysed with GAMs.

Results

We did not find any difference between survival of woodlice from the control group when compared with woodlice from the polish-marked group (3 *vs* 1 dead individual in these groups; p=0.615) or with woodlice from the marker-marked group (3 *vs* 0 dead individuals; p=0.241).

In total, 8640 records of behaviour were collected, but some behaviour categories were recorded rarely (*cleaning* 25 times, *interacting* 37 times, *monitoring* 88 times). Influence of marking on behaviour was evident in most cases at first sight: animals looked apathetic (i.e. they moved slowly and were less disturbed during manipulations than the controls).

There are differences evident between *activity* of woodlice from control group and woodlice from both marked groups (Fig. 1). Woodlice were active mainly dur-

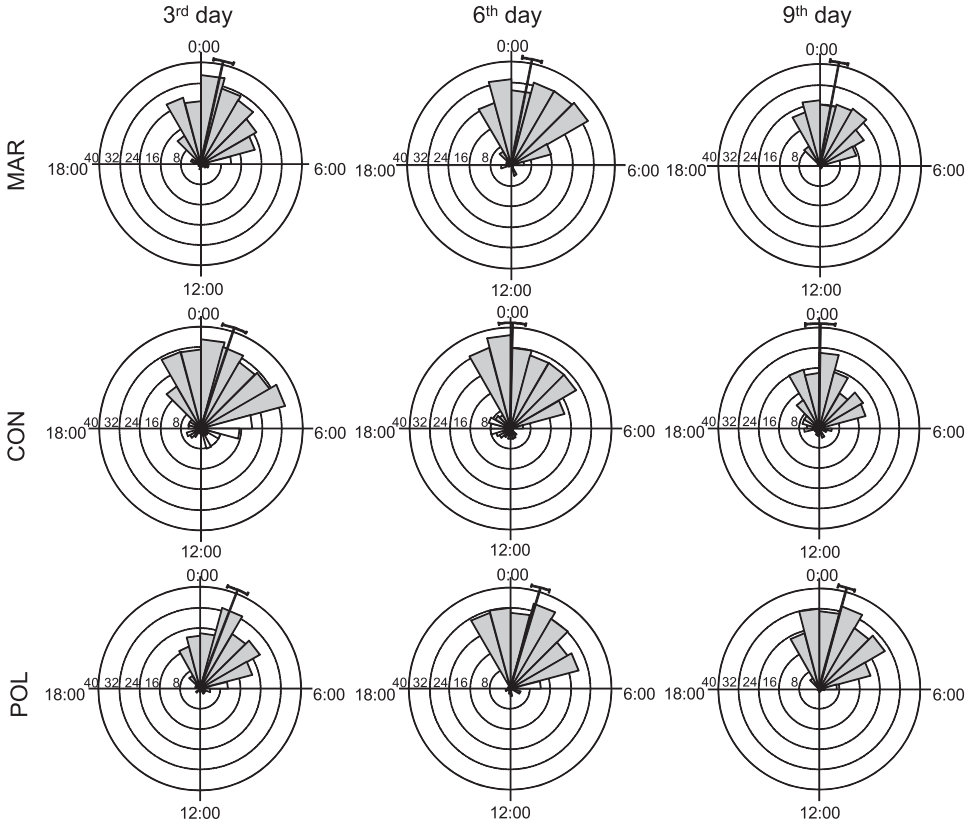

Figure 1. Time-distribution of active behavioural categories (feeding and/or exploring) of *A. vulgare* from all groups in observational days. Legend: CON – control, MAR – marker-marked, POL – polish-marked, grey triangles mark night-time activity, black line running from the centre of the diagram to the outer edge marks mean time of activity and the arcs extending to either side represent the 95% confidence limits.

ing night, although a few unmarked individuals were active during the daylight as well. Their activity generally started between 21:00 and 22:00 and finished at 05:00. Peaks of activity were between 00:00 and 01:30 (Fig. 1). Activity of woodlice from both marked groups was significantly lower in all observation days (with the exception of polish-marked group in the last day, Table 1) and showed the same daily pattern.

All main behavioural categories were recorded with a significant 24 hour pattern. The typical daily patterns of behavioural categories of *A. vulgare* were visualized without effect of marking and effect of experimental day using GAMs (Fig. 2).

Resting of woodlice was recorded mainly before sunrise (c. 05:00–06:00, Fig. 2). Woodlice from both marked groups rested significantly less during the whole experiment (Figs 3a–c, Tab. 1). Resting was the least frequent behaviour category among evaluated ones; woodlice were recorded resting 846 times. *Feeding* was generally the second most frequented category (1023 recorded acts) of behaviour, woodlice fed regularly at c. 00:00–05:00 (Fig. 2). Nevertheless feeding was significantly decreased by marking; individuals from both marked groups fed less in contrast to unmarked ones in all three days (Figs 3d–f, Tab. 1). *Exploring* behaviour

Table 1. Statistical tests for each level of treatment that the estimate differs from zero. Whereas parameter estimate for control group was estimated as intercept, parameters for level marker and polish represent pure effects. Significance testing was carried out after accounting for variation imposed by time of day. Behavioural category activity represents joined evaluation of both active categories (i.e. feeding and exploring) (see Fig. 1).

		activity		resting		feeding		exploring		hiding	
		z value	*p*	*z value*	*p*	*z value*	*p*	*z value*	*p*	*z value*	*p*
3rd day	*control* (intercept)	-9.30	< 0.001	-17.87	< 0.001	-17.17	< 0.001	-19.33	< 0.001	-4.68	< 0.001
	marker (x *control*)	-6.43	< 0.001	-8.52	< 0.001	-4.51	< 0.001	-1.45	0.147	12.34	< 0.001
	polish (x *control*)	-8.91	< 0.001	-5.22	< 0.001	-6.09	< 0.001	-2.64	0.008	11.93	< 0.001
6th day	*control* (intercept)	-11.93	< 0.001	-19.50	< 0.001	-16.58	< 0.001	-15.69	< 0.001	0.77	0.444
	marker (x *control*)	-4.48	< 0.001	-2.61	0.009	-3.18	0.001	0.09	0.932	6.00	< 0.001
	polish (x *control*)	-3.34	< 0.001	-5.33	< 0.001	-5.30	< 0.001	3.75	< 0.001	7.18	< 0.001
9th day	*control* (intercept)	-15.06	< 0.001	-19.15	< 0.001	-16.88	< 0.001	-19.06	< 0.001	7.59	< 0.001
	marker (x *control*)	-4.49	< 0.001	-4.83	< 0.001	-4.19	< 0.001	-1.02	0.306	7.39	< 0.001
	polish (x *control*)	-1.12	0.264	-4.67	< 0.001	-4.42	< 0.001	3.22	0.001	4.45	< 0.001

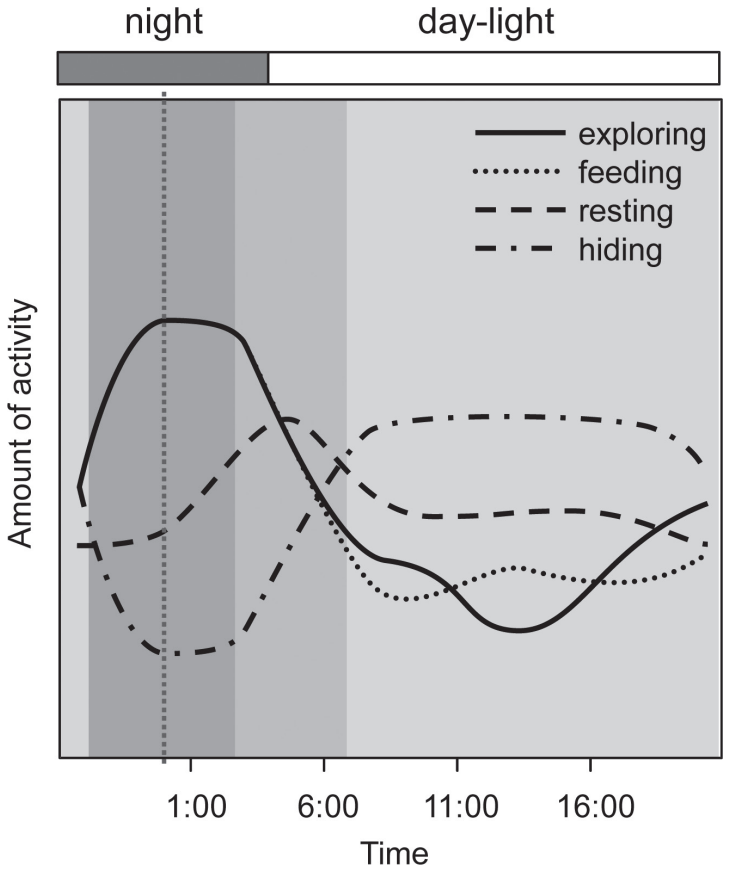

Figure 2. Daily patterns of behavioural categories as modelled by fitting GAM to illustrate a high degree of non-linearity in the response (logits). Compound graph from curves expressing frequency of exploring, feeding, resting and hiding of *A. vulgare* in a mean day

of woodlice (recorded 982 times) showed a typical and significant daily pattern in spite of marking; woodlice were exploring boxes during night and feeding at the same time (Fig. 2). Although there were no significant differences in the frequency of exploring between woodlice from marker-marked group and woodlice from control group, woodlice marked by nail polish exhibited significantly less exploring in the 3rd day and more exploring in following days (Figs 3g–i, Table 1). *Hiding* was the most frequent behaviour (5523 recorded acts). Woodlice were hidden especially during daylight (c. 06:00–21:00, Fig. 2). Marked woodlice were hidden in shelters significantly and strikingly more frequently compared with unmarked woodlice (Figs 3j–l, Table 1).

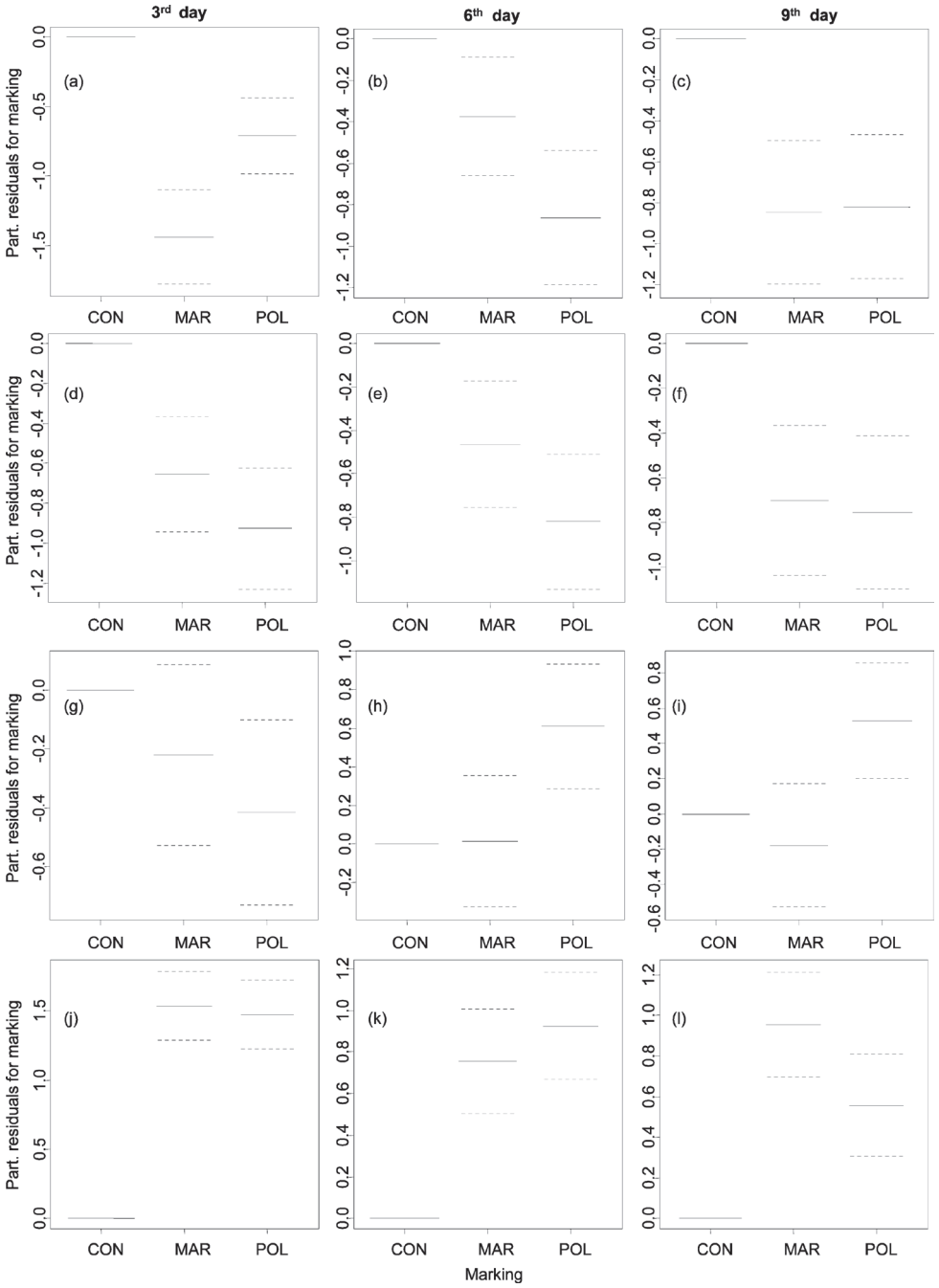

Figure 3. Influence of marking on frequency of *resting* (a), (b), (c), on *feeding* (d), (e), (f), on *exploring* (g), (h), (i), and on *hiding* (j), (k), (l) of *A. vulgare* in 3rd, 6th and 9th day analyzed by GAMs (confidence intervals dotted). Legend: CON – control, MAR – marker-marked, POL – polish-marked.

Discussion

We evaluated the effect of two external marking agents (nail polish and queen-bee marker) on behaviour and survival of the common pill woodlouse *Armadillidium vulgare*. Neither agent had any effect on survival of woodlice, but influence on behaviour was evident in almost all studied cases. Woodlice of both marked groups were less active, with less feeding and more hiding in contrast to those from the control group. Woodlice marked by nail polish also exhibited less exploring at 3rd day.

Den Boer (1961) used marking of woodlice (*Porcellio scaber* Latreille, 1804) by "shellac-solution in alcohol with pigment" to study shelter fidelity. He marked woodlice found on trees and tried to observe them again an hour later. He saw only about 10–20% of them (even though he prevented them escaping from the trees using tree-banding grease) and he concluded that the marked woodlice were hidden in shelters on tree trunks. Similarly our marked woodlice from both groups exhibited more hiding over the whole experiment. Their hiding behaviour could be connected with aggregation as result of attraction between conspecifics (Devigne et al. 2011) as well as looking for excrement as suitable source of food (Hassall and Rushton 1982). Greater exploring behaviour of unmarked woodlice at start of experiment can be associated with active interest in the new neighbourhood, marked animals were more apathetic.

Paris and Pitelka (1962) using marked woodlice (*A. vulgare*) found that the population is very fluid. They observed only a few marked individuals in bait traps the day after marking. At first sight, this is contrary to our results. Nevertheless from the activity pattern of *A. vulgare* it is evident that they are hiding during daylight and feeding/exploring during night. Paris and Pitelka checked their traps during nights, i.e. during feeding/exploring. Probably the marked animals were hidden somewhere else and did not enter trap due to lower activity and lower level of feeding.

Common pill woodlice were significantly less active due to marking. Cuticle of terrestrial isopods is relatively permeable to water, they avoid desiccation by finding a locality with suitable humidity, e.g. shelter during daytime (Hornung 2011). In our parallel study with the pill millipede, *Glomeris tetrasticha* Brandt, 1833, marked individuals were also significantly less active than unmarked ones. Moreover, this effect of marking on activity was much more intensive compared with the results presented here about *A. vulgare* (Drahokoupilová and Tuf 2011). Perhaps we could search for the reasons in anatomy. The thin cuticle of *G. tetrasticha* is very permeable for water (Edney 1951) in comparison with thicker cuticle of *A. vulgare*. We suppose some chemicals from polish and marker might break through cuticle into haemolymph of pill woodlice as well as pill millipedes. Lower activity and higher resting could have been a result of some poisoning overshoot. This question should be explored. The queen-bee marker probably did not affect behaviour of marked bees, because the dot of marking agent is not in contact with cuticle but usually only with hairs (Sammataro and Avitabile 1978). The lack of evidence for effect of marking on survival of woodlice should be interpreted carefully. Firstly, we evaluated effect of marking on survival and behaviour for 9 days only. We do not know if marked woodlice will show higher mortality later

or not. Late increased mortality could be caused e.g. by reduced feeding activity of marked woodlice. Secondly, we found an effect of external marks of nail polish on survival of woodlouse *P. scaber* in a longer experiment recently (Tuf et al., in prep.).

Our observations about night activity of *A. vulgare* are supported by previous studies. Refinetti (2000) found that *A. vulgare* shows strongly nocturnal activity under a natural light-dark cycle, more or less controlled by an endogenous timer (Cloudsley-Thompson 1956, Smith and Larimer 1979).

We conclude that common pill woodlice should not be externally marked by nail polish or by queen-bee marker. Both marking agents cause lower activity of marked woodlice and their usage, for example in capture-mark-recapture studies, can provide biased or wrong results.

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Our research was methodologically inspired by a nice lecture of Aline Quadros (Universidade Federal da Integração Latino-Americana, Foz do Iguaçu, Brasil). We would like to thank Emil Tkadlec (Palacky University, Olomouc, Czech Republic) for his introduction to R program. Alena Dvorská and Megan Short (Deakin University Melbourne, Australia) kindly checked English in previous versions of the manuscript. We are also grateful to two anonymous reviewers for valuable comments on the manuscript.

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RESEARCH ARTICLE

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Tonic immobility in terrestrial isopods: intraspecific and interspecific variability

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Abstract

Many arthropods, including terrestrial isopods, are capable of entering a state of tonic immobility upon a mechanical disturbance. Here we compare the responses to mechanical stimulation in three terrestrial isopods *Balloniscus glaber*, *B. sellowii* and *Porcellio dilatatus*. We applied three stimuli in a random order and recorded whether each individual was responsive (i.e. showed tonic immobility) or not and the duration of the response. In another trial we related the time needed to elicit tonic immobility and the duration of response of each individual. *Balloniscus sellowii* was the least responsive species and *P. dilatatus* was the most, with 23% and 89% of the tested individuals, respectively, being responsive. Smaller *B. sellowii* were more responsive than larger individuals. *Porcellio dilatatus* responded more promptly than the *Balloniscus* spp. but it showed the shortest response. Neither sex, size nor the type of stimulus explained the variability found in the duration of tonic immobility. These results reveal a large variability in tonic immobility behavior, even between closely related species, which seems to reflect a species-specific response to predators with different foraging modes.

Keywords

Death-feigning, anti-predator strategies, Crinocheta

Introduction

To be successful in avoiding their predators, preys engage in a multitude of behaviors, such as the construction of shelters (Manicom et al. 2008), decrease in activity and change in activity period according to their predator's (Sakamoto et al. 2006), change

of foraging sites to decrease predator encounter chances (Lima and Dill 1990) and even foraging in sites that will allow a better chance of escape (Wirsing et al. 2007). However, predators have their strategies too, which often leads them to their prey. Once in close contact with a predator, prey may engage in a second class of anti-predator strategies, i.e. to avoid being captured and/or being consumed. Amongst this class we find behaviors such as autotomy, release of chemical substances and tonic immobility (Langerhans 2007).

Tonic immobility is a state of reversible physical immobility and muscle hypertonicity, during which the organism lack responsiveness to external stimulation (Gallup 1974). It is a widespread form of passive anti-predator behavior employed by a variety of animals such as reptiles (Santos et al. 2010), harvestmen (Machado and Pomini 2008), orthopterans (Nishino and Sakai 1996, Faisal and Matheson 2001), coleopterans (Miyatake et al. 2004), hymenopterans (King and Leaich 2006) and crustaceans (Holmes 1903, Saxena 1957, Bergey and Weis 2006, Scarton et al. 2009). Tonic immobility is often called thanatosis or death-feigning, but these terms may be misleading in some cases where animals engaging in tonic immobility often assume different positions than dead animals (see Honma et al. 2006). Tonic immobility has been intensely addressed experimentally, especially in arthropods (Prohammer and Wade 1981, Honma et al. 2006, Miyatake et al. 2009, Nakayama and Miyatake 2010). It has been show to function as a defense mechanism and have an adaptive significance in several situations. One is when immobility physically impedes consumption. Honma et al. (2006) demonstrated that the consumption of the grasshopper *Criotettix japonicus* (Haan) by its frog predator *Rana nigromaculata* Hallowell was reduced because the posture assumed during tonic immobility enlarged the grasshopper's functional size and this was effective against the gape-limited predator (Honma et al. 2006). Other situations are when predators lose interest in unmoving prey (Miyatake et al. 2004) and when feigners are less likely to be preyed when in the presence of non-feigners (Miyatake et al. 2009). One common characteristic that emerges from these studies is that there is often a high intraspecific variability in tonic immobility behavior. This variability manifests both in relation to the responsiveness of the individuals and in the duration of their responses. It has been shown that both characteristics have a genetic basis (Prohammer and Wade 1981, Miyatake et al. 2004).

Amongst crustaceans, tonic immobility has been observed in crabs and terrestrial amphipods and isopods. Terrestrial isopods (Oniscidea) are preyed upon by a large variety of animals: spiders (Řezáč and Pekár 2007), chilopods (Sunderland and Sutton 1980), opiliones (Santos and Gnaspini 2002), ants (Dejean 1997), land flatworms (Prasniski and Leal-Zanchet 2009), amphibians and reptiles (Vitt et al. 2000, Van Sluys et al. 2001), among others. These predators employ a variety of prey-seeking and prey-handling behaviors. Naturally, terrestrial isopods have responded to this predation pressure by developing a variety of anti-predator strategies, which include a combination of behavioral and morphological traits (Schmalfuss 1984) and also a form of chemical defense which is unique among crustaceans (Gorvett 1956, Deslippe et al. 1996). According to behavioral and morphological traits related to predator avoidance,

isopod species can be grouped into ecomorphological groups, as proposed by Schmalfuss (1984): The "runners" are narrow, have long and strong pereopods adapted to run and escape fast when they are uncovered or disturbed. The "clingers", on the other hand, have broad tergites, short pereopods, and flattened bodies and when disturbed, tend to remain motionless and tightly attached to the substrate. The "rollers" are capable of entering a state of tonic immobility when disturbed, forming a near perfect or a perfect ball which encloses the pereopods and pleopods and hides the ventral surface of the animal. The "spiny forms" are also conglobating forms that have, in addition, conspicuous dorsal spiny protuberances (Schmalfuss 1984).

The conglobation ability of "rollers" and "spiny forms" is, for sure, a well-known example of tonic immobility in response to disturbance. As pointed by Saxena (1957) when referring to the roller *Armadillidium vulgare* Latreille "*a light pressure on the ventral side and sometimes a fall from a height of 2 or 3 inches will bring about this motionless state and induce the characteristic roll up into a ball like shape*". However, many other species of different ecomorphological groups, such as clingers (Schmalfuss 1984) and runners (Sokolowicz et al. 2008) are also capable of entering a state of tonic immobility upon disturbance. In such groups, tonic immobility involves the contraction of the body to form a comma-like shape 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 contour of the first pereonites (pers. obs.). Although tonic immobility is a widespread response in terrestrial isopods, it has not been addressed experimentally, except for the work of Saxena (1957). Here we compare the responses to mechanical stimuli of three species belonging to the clinger ecomorphological group. Two are close-related species: *Balloniscus glaber* Araujo & Zardo and *Balloniscus sellowii* (Brandt) (Balloniscidae) and the other is a Porcellionidae, *Porcellio dilatatus* Brandt. We ask the following questions: (1) Do the species differ in responsiveness to tonic immobility-inducing stimuli? (2) Does the responsiveness depend upon sex, size or stimulus? (3) Is the duration of tonic immobility influenced by sex, size or type of stimulus, and does it differs between species? (4) Is the duration of tonic immobility related to the time needed to elicit a response?

Methods

Species sampling and laboratory conditions

Leaf litter samples containing *B. glaber*, *B. sellowii* and *P. dilatatus* (Fig. 1) were collected in urban areas and vicinities of the campus of Universidade Federal do Rio Grande do Sul, in Porto Alegre, in the south of Brazil. *Balloniscus glaber* were captured in July 2008, and *B. sellowii* and *P. scaber*, in July and November 2009, respectively.

In the laboratory, we randomly pick 60 intermoult individuals (near 1:1 sex ratio) of each species and put them individually in Petri dishes (9 cm diameter) containing moist soil, food (decayed leaves) and plastic black shelters. They were left undisturbed

for 72 h prior to the trials. Ovigerous females or females with an empty marsupium were not used in the trials. The isopods were maintained side by side in a large shelve, about 1.30 m height, illuminated by phosphorescent light, in a room with temperature of 20°C and a 12:12 photoperiod. To minimize manipulation prior the trials, the individuals were measured (cephalothorax width) only at the end of the experiments.

Types of stimuli

We choose three different stimuli to be applied to the isopods: touch, squeeze and drop. These stimuli are known to elicit tonic immobility as they frequently did so when we are handling isopods of various species in the laboratory, and they could mimic the mode of action of different predators. The stimulus "touch" consisted of repeatedly pinching the isopod's body laterally with the tip of a metal forceps and gently pushing it. When inspecting soil and litter samples, it is very common to observe tonic immobility in the isopods after touching/pushing then (pers. obs.). The stimulus "squeeze" consisted of grabbing the isopod with a forceps and slightly squeezing it, in an attempt to simulate the bite of an ant. The "drop" stimulus consisted of grabbing the isopod with a forceps, lifting it approx. 10 cm and then letting it drop in the dish. Here we were trying to mimic a larger predator, such as a small bird or lizard, letting the isopod to fall after grabbing it. The experiments that will be described below were conducted always by two observers, and the observer that applied the stimuli to the isopods was the same throughout all the trials. Care was taken to apply the stimuli and not causing injuries to the isopods. In fact, seven *P. dilatatus* and one *B. sellowii* died during the acclimation period but there was no mortality during the experiments.

Experiment 1

To answer questions 1, 2 and 3 we applied the three different stimuli described above to each individual in a random sequence. Each species was tested on a separate day, and all individuals of the same species were tested on the same day. Before starting the trials, the shelters and leaves were removed from all petri dishes to allow a proper observation of isopod behavior.

We started the trial by randomly picking a number corresponding to an individual and a number corresponding to a stimulus to be applied to that individual (e.g. 1 for touch, 2 for squeeze, 3 for drop). Then, we removed the lid of the petri dish and applied the stimulus to the isopod. The stimulus was repeated up to 3× in case of "squeeze" or "drop" or 5× in case of "touch". If the stimulus did not elicited tonic immobility, the individual was considered non-responsive. If the individual responded, i.e. showed the characteristic posture of tonic immobility (see Fig. 1), the duration of tonic immobility was recorded with a stopwatch. The end of the response was when the individual showed any slight movement, which usually began with a movement of the antenna.

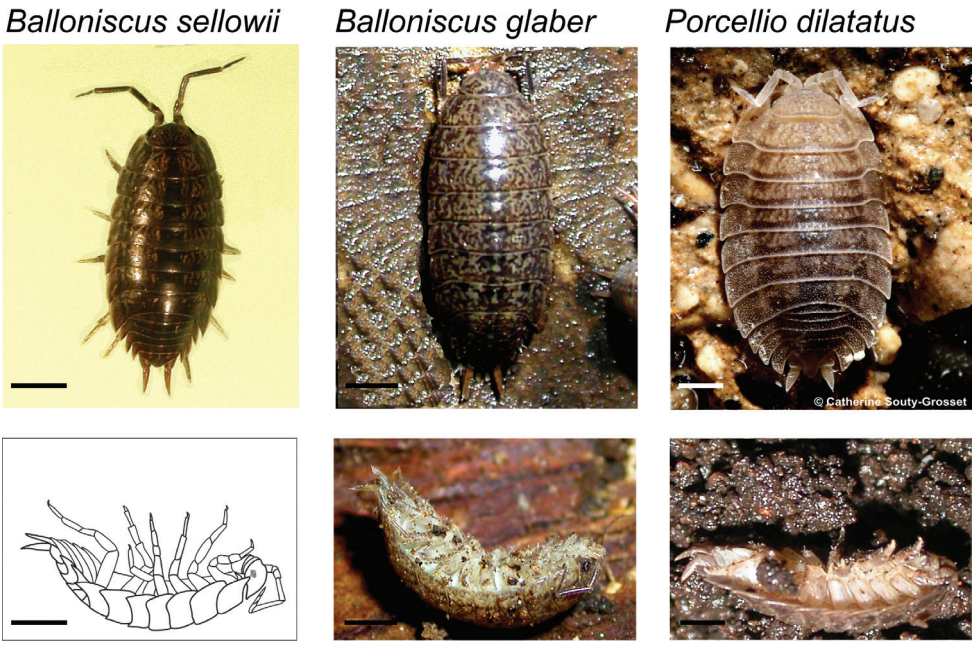

Figure 1. Terrestrial isopods studied in dorsal view: *Balloniscus sellowii*, *B. glaber* (Balloniscidae) and *Porcellio dilatatus* (Porcellionidae) (top) and their respective postures during tonic immobility (bottom). For *Balloniscus sellowii* a drawing made from a photograph is presented. Bars = 2 mm.

The lid was placed again on the dish and another number was randomly picked. These procedures were repeated until all individuals have been subject to the three stimuli.

Experiment 2

To answer question 4 we conducted a second experiment, on the day after experiment 1, with the same individuals. The order of the individual was defined randomly as in the experiment 1. We removed the lid of the dish and stressed the isopod with the tip of a forceps for up to 30 seconds, i.e. applying only the "touch" stimulus. If during this period the stimulus did not elicit tonic immobility, the individual was considered non-responsive. If the isopod responded, it was recorded both the time it took to respond (to enter the posture) and the duration of response.

Analyses

Proportion data from experiment 1 was compared with a G test of independence (for two samples) or a chi-square test (one sample). The relationship between individual size and its responsiveness was investigated with a logistic regression. To answer ques-

tion 3, the differences in the duration of tonic immobility among species and stimuli was tested with ANOVA and Tukey test. Also for each species the relationship between duration and size was tested with ANCOVA using sex as the co-variable. Finally, to answer question 4, a linear regression was applied to verify if there was relationship between the time to elicit tonic immobility and duration of response (experiment 2). For all tests the duration of response was log-transformed. All tests were made using the R© Software v. 2.13.0 (The R Foundation for Statistical Computing 2011).

Results

Experiment 1

Do the species differ in responsiveness?

Porcellio dilatatus and *B. glaber* differed remarkably from *B. sellowii* (G=62.26; p<0.0001) (Fig. 2A): *Porcellio dilatatus* and *B. glaber* were the most responsive species and did not differ from each other; 89% and 78% of their individuals, respectively, were responsive to at least one of the stimuli applied; 51% of the responsive *P. dilatatus* individuals were responsive to all three stimuli applied, while only 12% of *B. glaber* individuals' were so. *Balloniscus sellowii* differed from both species in that only 23% of the tested individuals were responsive to at least one stimulus and none responded to all three stimuli (Fig. 2A).

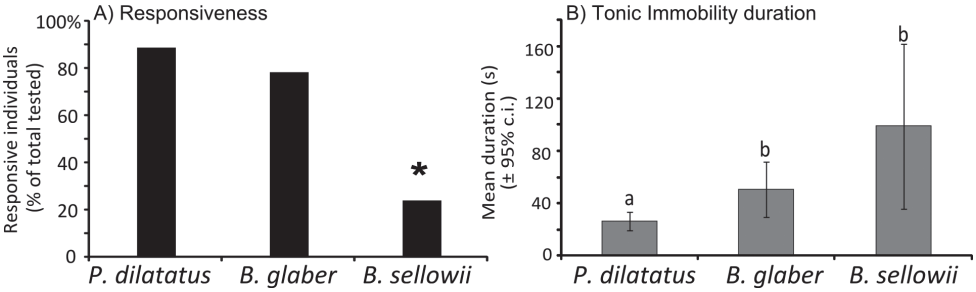

Figure 2. Responsiveness and tonic immobility duration in terrestrial isopods. **A** Percentage of responsive individuals in the three species tested. The * indicates a significant difference between species, after a G-test. **B** Mean tonic immobility duration in seconds for each terrestrial isopod (considering all stimuli pooled) in experiment 1. Different letters indicate significant differences, after ANOVA and Tukey test.

Does the responsiveness change according to the sex, stimuli and size?

Regarding the different stimuli, *P. dilatatus* was responsive to the three stimuli equally (χ^2=1.75; p=0.417), whereas *B. glaber* was more responsive to "drop" (χ^2=11.703; p=0.003) and *B. sellowii* was more responsive to "touch" (Fig. 3A) (χ^2=7.882; p=0.019).

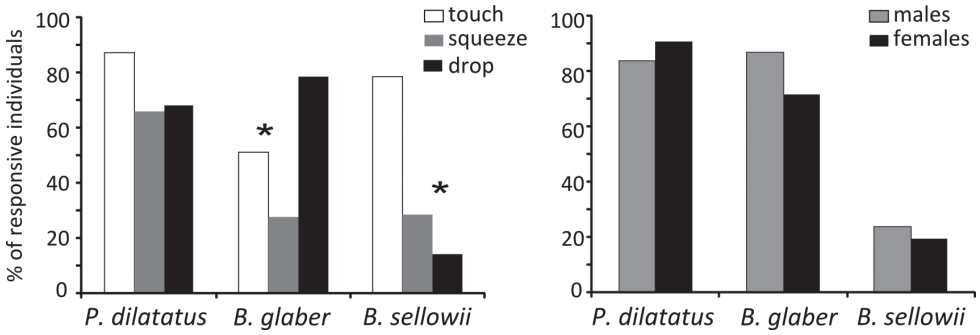

Figure 3. A Percentage of responsive individuals to each specific stimulus, in relation to the total number of responsive individuals of each species. **B** Percentage of responsive males and females in relation to the total number of males and females of each species tested. The * indicates a significant difference between stimuli, after a χ^2 test.

The proportion of responsive males and females did not differ in all species (*P. dilatatus* χ^2=0.111; *p*=0.73; *B. glaber* χ^2=0.812; *p*=0.36; *B. sellowii* χ^2=0.008; *p*=0.92) (Fig. 3B).

In relation to size, an interesting trend was observed. In *P. dilatatus* and *B. glaber*, responsiveness was independent of individual size. As mentioned above, most individuals of these species were responsive (Fig. 4). In *B. sellowii*, however, there were intraspecific differences in responsiveness: a fitted logistic regression indicated that the probability of being responsive is higher in younger individuals and decreased with size (=age) (Fig. 5) (Logistic regression: Model intercept=3.254; s.e.=1.39; *p*=0.019; Size (factor) = -2.821; s.e.= 0.951; *p*=0.003; AIC=60.21).

Is the duration of tonic immobility influenced by the species, stimulus, sex, or size?

The duration of tonic immobility was highly variable in all trials: it ranged from a few seconds up to 3 min in *P. dilatatus*, 7 min in *B. sellowii* and up to 12 min in *B. glaber*. The duration of response differed between species (Fig. 2B), but the effect of the different stimuli was not significant (Table 1). *Porcellio dilatatus* remained in tonic immobility for a shorter time interval than the two *Balloniscus* species (Fig. 2B).

Using ANCOVA we verified that neither sex nor size of the isopods explained the variation found in the duration of tonic immobility (Table 2).

Table 1. Differences in the duration of tonic immobility depending on the species and different stimuli.

	ANOVA results				
Factors	d.f.	SS	MS	*F*	*p*
Species	2	8.91	4.45	13.36	*p*<0.001
Stimuli	2	0.93	0.46	1.39	*p*=0.250
Residuals	190	63.34	0.33		

Table 2. Relationship between the duration of tonic immobility and individual size (cephalothorax width in mm) and sex (co-variable).

ANCOVA results				
Factors	d.f.	SS	*F*	*p*
	Porcellio dilatatus			
Size	1	0.143	0.587	0.44
Sex	1	0.836	3.426	0.07
Size:Sex	1	0.147	0.602	0.44
Residuals	37	9.035		
	Balloniscus glaber			
Size	1	0.052	0.226	0.63
Sex	1	0.287	1.248	0.27
Size:Sex	1	0.019	0.085	0.77
Residuals	27	6.215		
	Balloniscus sellowii			
Size	1	0.366	1.127	0.30
Sex	1	0.514	1.582	0.22
Size:Sex	1	0.516	1.583	0.22
Residuals	16	5.205		

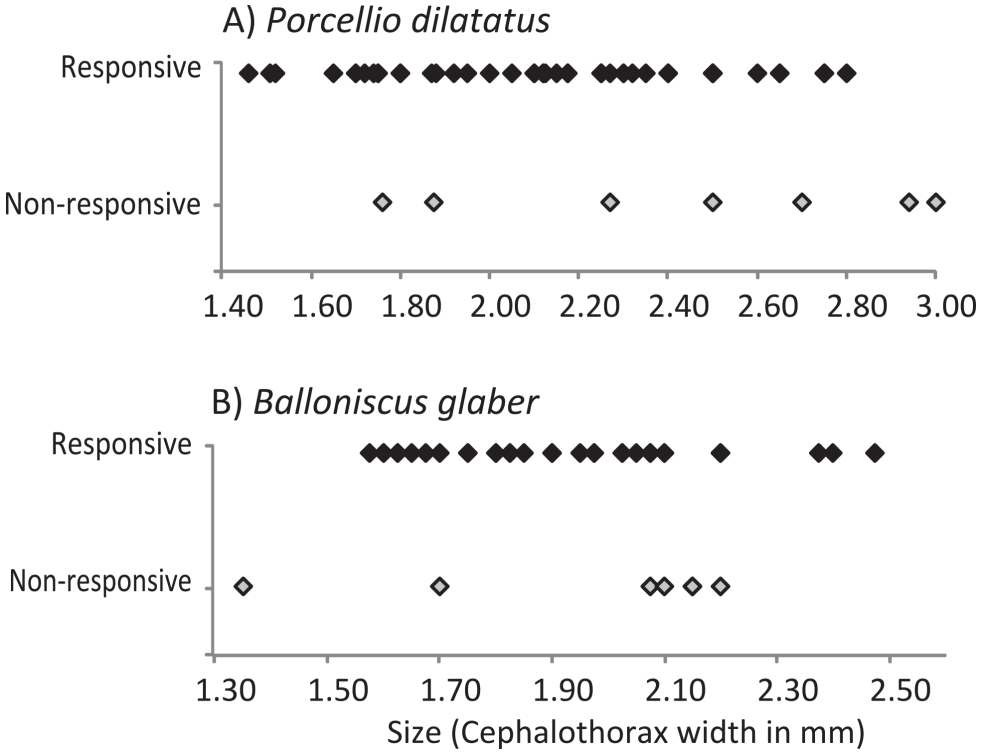

Figure 4. Size and response to the stimuli in **A** *Porcellio dilatatus* and **B** *Balloniscus glaber*. Responsive individuals are represented with black marks and non-responsive individuals with grey marks.

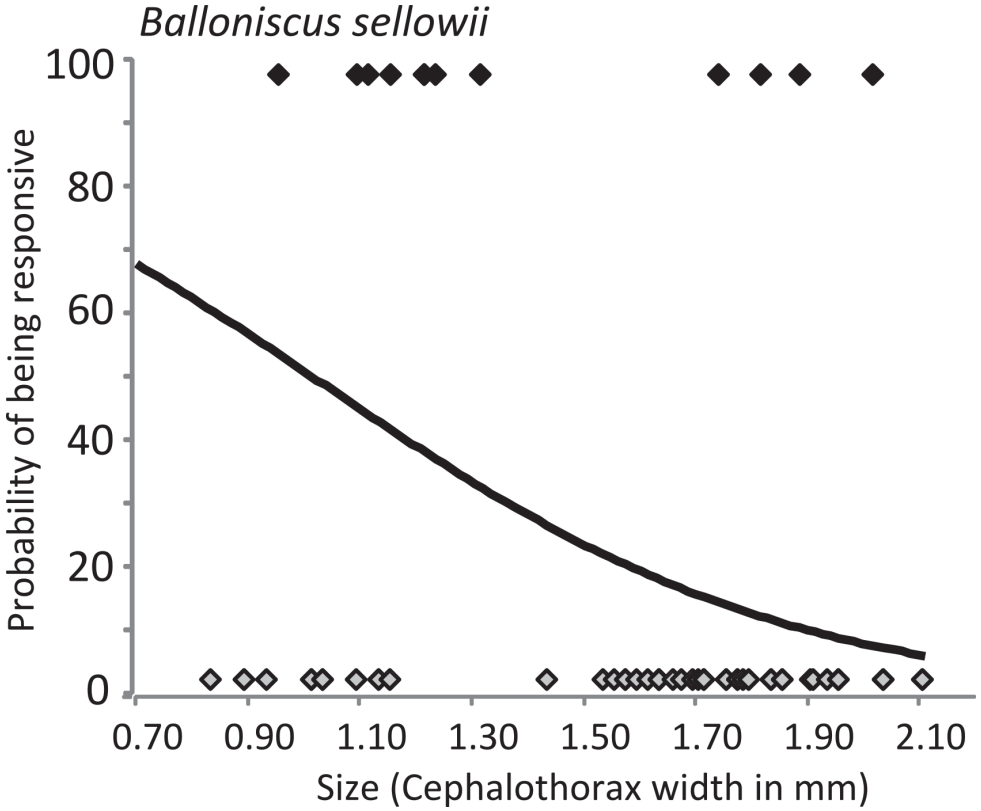

Figure 5. Responsiveness of *B. sellowii* individuals in relation to size. The line models the probability of being responsive according to the individual size (after a logistic regression). The black and grey symbols show the responsive and non-responsive individuals, respectively.

Experiment 2

Is the duration of tonic immobility related to the time needed to elicit a response?

In this experiment, 77% *P. dilatatus,* 65% *B. glaber* and 34% *B. sellowii* individuals responded to the stimulus within the 30 seconds. Upon visual inspection of data in Fig. 6 it can be seen that for none of the species there was a relationship between the time elapsed until tonic immobility and the duration of the response, which was confirmed with the linear regression analysis (see Fig. 6). In the case of both *Balloniscus* species there were individuals that responded promptly (in less than 5 s) or took more than 20 s to respond and remained in tonic immobility for short time intervals (less than 10 sec) and long time intervals (4 to 5 min). In all species tonic immobility duration was highly variable among individuals and it was independent of the time that each individual took to respond (Fig. 6). However, it can be noted a different pattern of response in *P. dilatatus*: 99% of the responsive individuals responded within 7 s of stimulation (Fig. 6).

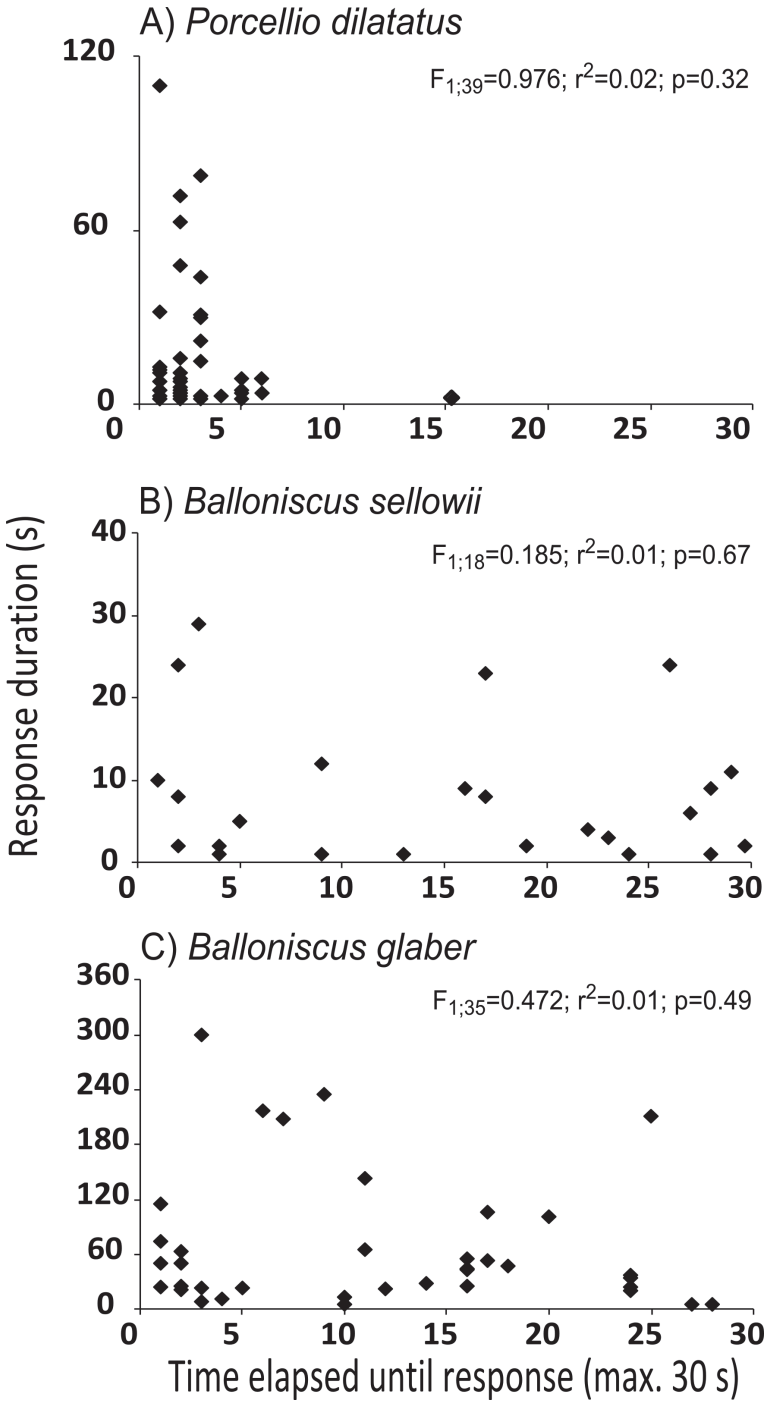

Figure 6. Relationship between the time elapsed until the beginning of tonic immobility and the duration of response, for responsive individuals in experiment 2. The values indicate the results of the linear regression analysis.

Discussion

In this study we used two intrinsic factors (sex and size of the individuals) and one extrinsic factor (different stimuli) to try to explain the intraspecific variability found with respect to the responsiveness and duration of the tonic immobility showed by terrestrial isopods. We found an influence of the size in the responsiveness of *B. sellowii* and an influence of the type of stimulus in both *Balloniscus* species. Regarding the duration of tonic immobility, none of the factors explained the variability exhibited by the individuals. These findings are discussed below in more detail.

Males and females of different species are known to differ in responsiveness and duration of tonic immobility. For instance, in the beetle *Callosobruchus maculatus* (Fabricius) the females had a significantly higher frequency and longer duration of tonic immobility than males (Myatake et al. 2008a). In the freshwater crab *Trichodactylus panoplus* von Martens females stayed immobile for longer time than males (Scarton et al. 2009). In the terrestrial isopods studied here there was no indication of any sex-related differences in tonic immobility behavior, and maybe this indicates that both males and females are exposed to the same predators.

Upon encounter with a predator, a prey may run or enter tonic immobility, but it cannot adopt both strategies at the same time (Ohno and Miyatake 2007). In fact, there are many examples in the literature showing that prey specializes in one type of strategy at the expense of the other (Miyatake 2001a). King and Leaich (2006) found a negative relationship between tonic immobility and locomotor activity in the parasitoid wasp *Nasonia vitripennis* Walker. Also, a negative genetic correlation between tonic immobility intensity and locomotor activity (Nakayama and Miyatake 2010) was demonstrated in the beetle *Callosobruchus chinensis* (L.), both for field populations and populations artificially selected for either tonic immobility intensity or flying ability (Ohno and Miyatake 2007). Here we observe that, in contrast to the other species, *B. sellowii* does not engage in tonic immobility very often, and their smaller (= younger) individuals were more likely to do so than the larger (= older) individuals. Based on that observations, we propose that *B. sellowii* may change its anti-predator behavior along its lifetime, employing tonic immobility more often when young and small and adopting a more active escape strategy, as running, when older (= larger). In the same line, days-old workers of the ant *Solenopsis invicta* Buren responded to intraspecific aggression with tonic immobility, however when months-old the workers responded to the same aggressors with a more active response, by fighting back (Cassill et al. 2008). This strategy, employed against aggressive conspecific ants, is effective because days-old workers have a relative soft exoskeleton and at this stage tonic immobility increase their survivorship (Cassill et al. 2008).

Among the extrinsic factors that are known to influence responsiveness to tonic immobility-inducing stimuli, temperature (Miyatake et al. 2008a), light (Saxena 1967, Miyatake 2001a) and starvation (Miyatake 2001b) have been stressed. In addition to that, here we demonstrated that the type of stimulus can also influence responsiveness. Although there was no record in the literature of tonic immobility being shown by isopods upon a encounter with a predator, it can be hypothesized that the characteristic

posture observed during tonic immobility by terrestrial isopods increase survivorship in two ways: it could increase resemblance with the substrate and make the animal less conspicuous to a visual predator (if compared to a moving prey) (Bergey and Weis 2006) and/or it could difficult the predator's access to its vulnerable ventral surface. In this last scenario, tonic immobility could be effective against an attack by a small invertebrate that capture prey by biting or stinging, such as spiders or ants. By responding more to "drop" than to the other stimuli *B. glaber* seems to be responding to visual predators larger than an isopod, such as reptiles, that possibly lose their prey, in which case they would enter tonic immobility and foil the predator. On the other hand, *B. sellowii* responded more to "touch", which could indicate a response to a smaller biting predator and the isopod would be protecting its ventral body parts. These predictions need, however, further testing.

Tonic immobility duration varies widely intraspecifically. For instance, Machado and Pomini (2008) reported that individuals of the harvestmen *Camarana flavipalpi* Soares can remain immobile for 8 s to nearly 11 min, and *Hoplobunus mexicanus* (Roewer) from 21 s to 31 min (Pomini et al. 2010). Bergey and Weis (2006) noted that fiddler crabs can remain immobile for more than two hours, but on average duration ranges from 45 to 171 s. High intraspecific variability in the duration of tonic immobility was also evidenced here, especially for *B. glaber* and *B. sellowii*. In the present study, none of the intrinsic and extrinsic factors we tested explained the variability in the species studied. The duration of response is clearly associated with prey survival. Arduino and Gould (1984) indicate that animals engaging tonic immobility could be monitoring the environment for an opportunity to escape, so the variability observed in the duration of responses could reflect the time the individuals are waiting for the risk around them to decrease. Miyatake et al. (2004) demonstrated that beetles *Tribolium castaneum* (Herbst) selected for long duration of tonic immobility were less prone to predation than those selected for short duration after exposing them to the attack of a Salticidae spider, *Hasarius adansoni* Audouin (Miyatake et al. 2004). Recent studies with insects (Miyatake et al. 2008b, Nishi et al. 2010) and spiders (Jones et al. 2011) indicate that the neurotransmitters dopamine and serotonin are involved in the regulation of tonic immobility duration.

In conclusion, we observe three different patterns of tonic immobility among the clingers studied. *Balloniscus sellowii* enters tonic immobility rarely, it is more likely to adopt an active defense, such as running. *Balloniscus glaber* is more responsive, irrespective of the stimulus applied, the sex or size of the individuals, and can stay in tonic immobility for a long time, which indicates that tonic immobility could be an important strategy for this species. *Porcellio dilatatus* is highly responsive, also irrespective of the stimulus applied, the sex or size of the individuals, but it shows a much shorter response. As pointed by Honma et al. (2006), to understand the evolutionary dynamics of prey interactions with their predators it is necessary to account for both the foraging mode of the predator and the predator-avoidance mode of the prey. Experiments with the predators of isopods are needed to investigate the effectiveness of tonic immobility and the significance of the intraspecific variability to survivorship.

In the terrestrial environment, isopods can be preyed upon practically all invertebrate and vertebrate carnivores and omnivores. It seems that the multitude of anti-predator strategies they have (tonic immobility, secretion of adhesive substance, development of spiny tergites, long pereopods for running) could be a response to a history of a high predation pressure. More studies, however, are needed to investigate whether these strategies indeed improve survivorship upon encounter with a predator, and to elucidate to which predators each response works.

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RESEARCH ARTICLE

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Terrestrial isopod community as indicator of succession in a peat bog

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Abstract

Terrestrial isopods were studied in the Dubravica peat bog and surrounding forest in the northwestern Croatia. Sampling was conducted using pitfall traps over a two year period. Studied peat bog has a history of drastically decrease in area during the last five decades mainly due to the process of natural succession and changes in the water level. A total of 389 isopod individuals belonging to 8 species were captured. Species richness did not significantly differ between bog, edge and surrounding forest. High species richness at the bog is most likely the result of progressive vegetation succession, small size of the bog and interspecific relationships, such as predation. With spreading of *Molinia* grass on the peat bog, upper layers of *Sphagnum* mosses become less humid and probably more suitable for forest species that slowly colonise bog area. The highest diversity was found at the edge mainly due to the edge effect and seasonal immigration, but also possibly due to high abundance and predator pressure of the *Myrmica* ants and lycosid spiders at the bog site. The most abundant species were *Trachelipus rathkii* and *Protracheoniscus politus,* in the bog area and in the forest, respectively. Bog specific species were not recorded and the majority of the species collected belong to the group of tyrphoneutral species. However, *Hyloniscus adonis* could be considered as a tyrphoxenous species regarding its habitat preferences. Most of collected isopod species are widespread eurytopic species that usually inhabit various habitats and therefore indicate negative successive changes or degradation processes in the peat bog.

Keywords

Edge, seasonal dynamics, pitfall trapping, predation, *Trachelipus rathkii*, *Protracheoniscus politus*, *Hyloniscus adonis*, tyrphoxenous species

Introduction

Peat bogs are a type of wetlands characterized by a high water table, low levels of nutrients, low pH values, and are dominated by *Sphagnum* mosses (Spitzer and Danks 2006). They are widely distributed in specific climatic regions where climates are cool and precipitation is relatively high, mostly in the boreal zones of Europe, Asia and North America (Rydin and Jeglum 2006, Spitzer and Danks 2006). In terms of biogeography, peat bogs in Croatia represent a southern enclave of their northern continuous distribution (Topić and Stančić 2006). Peat bogs are highly endangered ecosystems in Europe (Holmes et al. 1993, Dapkus and Tamutis 2008, Buchholz et al. 2009) and some are listed as priority habitat types in Annex I of the European Habitats Directive (Anonymous 1992). Due to their small size, isolation, drainage and abandonment of traditional human activities, and especially to the progressive vegetation succession, such habitats are among critically endangered habitats in Croatia (Topić and Stančić 2006, Topić and Vukelić 2009).

These unique ecosystems often consist of specialized flora and fauna, largely limited only to these habitats. Although most of the peat matrix is dead, it contains a great variety of living organisms that contribute in decomposing (Rydin and Jeglum 2006). According to Peus (1932) and Roubal (1934) species that inhabit peat bogs can be classified into four categories: (a) tyrphobiontic species (occur only in bogs), (b) tyrphophilous species (characteristic of bogs but not strictly confined to them), (c) tyrphoneutral species (distributed across various types of habitats) and (d) tyrphoxenous species (vagrants or immigrants that cannot live in bogs).

As members of the soil macrofauna community, isopods play an important role in the processes and soil formation (Wolters and Ekschmitt 1997). As soil humidity is their essential requirement, they occur in microhabitats such as under stones and logs in leaf litter (Schmalfuss 1978). There are several papers about isopod fauna in Central European wetlands (Sallai 1993, Farkas 1998, Tajovský 1998, Vadkerti and Farkas 2002, Tuf 2003, Vilisics et al. 2007). However, isopod fauna on peatlands is basically unknown, especially in Europe.

The objectives of this study were: (1) to determine the terrestrial isopod assemblages and their spatial distribution in the peat bog remnant and surrounding forest, (2) to determine which basic ecological groups inhabit the peat bog and adjacent forest (3) to analyze the seasonal dynamics of the dominant isopod species and (4) to asses possible influence of soil temperature and humidity on isopod abundance.

Methods

Study area

Peat bog Dubravica is located in the Northwestern part of Croatia, in Hrvatsko zagorje Region (45°57.430'N, 14°44.470'E). It is situated in sessile oak and hornbeam forest

(ass. *Epimedio-Carpinetum betuli* (Ht.1938) Borh. 1963) at an altitude of l60 m asl. Since 1966 it has been protected as a Botanical Reserve and it is a potential NATURA 2000 site.

According to Horvat (1939) there were three bogs that covered the area of 2566 m^2 in total, but during the last 50 years the bog area has drastically decreased in size. There is only one small peat bog remnant covering the area of 605 m^2 (Hršak 1996). This is mainly due to changes of the water level and abandonment of traditional management practices, such as mowing. All that led to the process of natural vegetation succession. A great part of the bog is overgrown by purple moor grass (*Molinia caerulea* (L.) Moench) and some woody species like the alder (*Alnus glutinosa* L.) and alder buckthorn (*Frangula alnus* Mill.). These changes in vegetation structure dry out and rise up the bog, enabling growth of less moisture substrate seeking plants (Hršak 1996). In order to preserve biodiversity of the peat bog remnant, the bog area is nowadays mowed once a year. Also, the presence of wild animals, like wild boar, brings to halting the spread of *Molinia* grass by rooting the land.

Three sites were selected in and around the bog area, situated in different vegetational associations (Figure 1). The site B is located in the centre of the bog and the bog vegetation belongs to the *Rhynchosporetum albae* W. Koch, 1926 association. The ground layer was dominated by typical bog plant species: common sundew (*Drosera rotundifolia* L.), white beak-sedge (*Rhynchospora alba* (L.) Vahl.) and *Sphagnum* mosses (*Sphagnum subsecundum* Ness.). Site E is located on the edge of the bog and sessile oak and hornbeam forest. The edge is covered with rather strong *Rubus* sp. plants, which are opportunistically taking the open area on the edge zones. In order to avoid edge effect, site F is located 60 m from the edge into the forest (Lindenmayer and Fischer 2006). This site belongs to *Epimedio-Carpinetum betuli* association. Sessile oak (*Quercus robur* L.) and hornbeam (*Carpinus betulus* L.) are the dominant species in a tree layer.

Sampling

Isopods were collected using pitfall traps during the carbide beetle study and sampling was conducted from May to November 2008 and from May to November 2009. Five plastic traps per site were placed (volume 0.3 dm^3) 5 m apart. They were half filled with a saturated solution of sodium chloride with a few drops of detergent to break the surface tension of the liquid. A styrofoam roof was placed above each trap to protect them from rain. The traps were emptied once a month. The collected specimens were kept in 75% ethyl-alcohol with glycerol. The individuals were identified to the species level, apart from *Armadillidium* sp. and Oniscidae sp., due to lack of adult male individuals. All individuals were identified by authors (I. A. and J. B.), with the exception of *Hyloniscus adonis*, which was identified by Stefano Taiti (Istituto per lo Studio degli Ecosistemi, Consiglio Nazionale delle Ricerche).

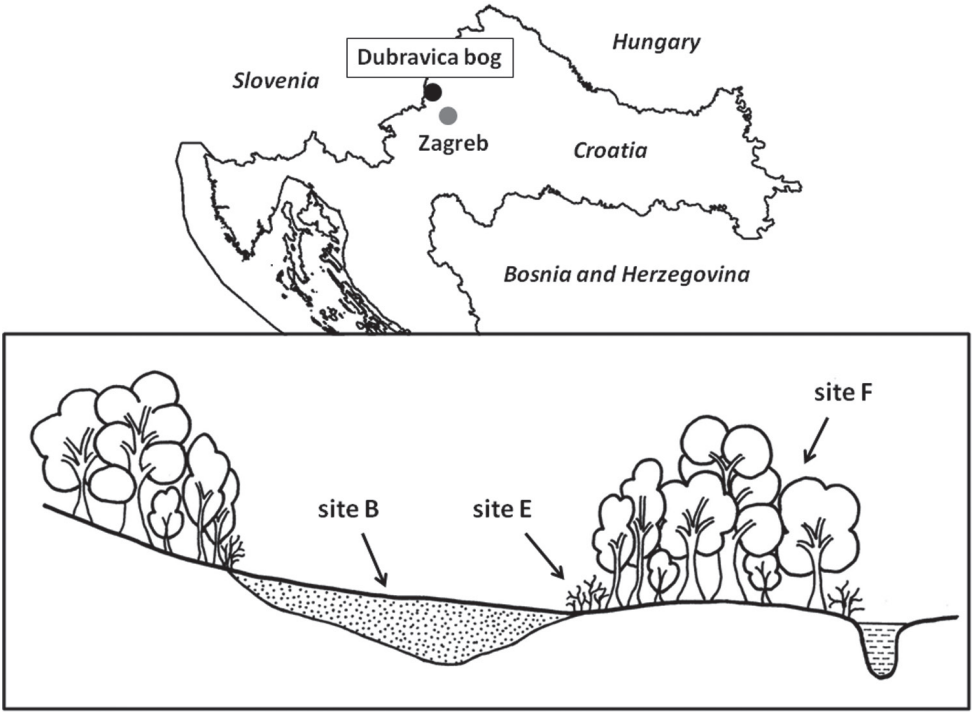

Figure 1. Sampling sites in the Dubravica bog and surrounding forest. **Site B** - bog **Site E** - edge **Site F** - forest.

Soil analysis

Basic pedophysiological properties of the soil were determined using standard methods (Škorić 1982). Soil temperature and humidity were measured once a month at all three sites, next to every pitfall trap and average values were calculated. Temperature was measured at depth of 7 cm using P300 Dostmann electronic thermometer and soil humidity after every field trip by taking a soil sample at approximately 10 cm. In substrate samples water content was determined by the gravimetric method. The same soil sample was used to measure pH in water with a ratio of 1:2.5 (w/v) (10 g substrate / 25 mL H_2O) and in KCl with a ratio of 1:2.5 (w/v) (10 g substrate / 25 mL KCl), using WTW pH 330i meter. Phosphorus, as P_2O_5 was measured by the colorimetric method using the spectrophotometer DR/2000 HACH (1996). Total nitrogen (TN) and total carbon (TC) content in substrate were simultaneously determined using the dry combustion method (elemental analysis) with the Vario Macro CHNS analyzer, Elementar.

Soil analysis showed that pH values were very low at each sampling site, while calcium carbonate concentration was lowest in the forest, but higher at the edge and at the bog site (Table 1). Soil humidity was highest (sometimes ≥80%) at the bog during most of the sampling period. In addition, comparable results were observed at the edge, where soil humidity was also high (≥70%) (Figure 2). Soil humidity was particularly low in the forest (18–33%) in both study years. Oscillations of the soil tem-

Table 1. Vegetational and pedagogical properties of studied sites in the Dubravica peat bog. **Site B** – bog; **Site E** – edge; **Site F** – forest.

Environmental variable	Site B	Site E	Site F
Vegetation analysis			
Plant association	*Rhynchosporetum albae* W. Koch	-	*Epimedio-Carpinetum betuli* (Ht 38) Both. 63
Vegetation height/m	1	0.2-0.5	20
Vegetation density	high	low	middle, thick layer of litter
Tree layer, dominant plant species	-	-	95% *C. betulus, Q. robur*
Frutescent layer, dominant plant species	0%	50% *Rubus* sp.	5% *C. betulus*
Herbaceous layer, dominant plant species	100% *S. subsecundum*, *M. caerulea*	10% *M. Caerulea*	80% *Epimedium alpinum*
Soil analysis			
Soil type	peat	-	stagnosol
pH (H_20)	4.47	4.18	4.09
pH (KCl)	3.92	3.55	3.57
Humus (Tjurin) %	7.0	6.3	2.0
P_2O_5 mg/100 g	0.8	1.7	0.4
$CaCO_3$ %	0.235	0.226	0.127
C/N	17.2	15.6	13.6

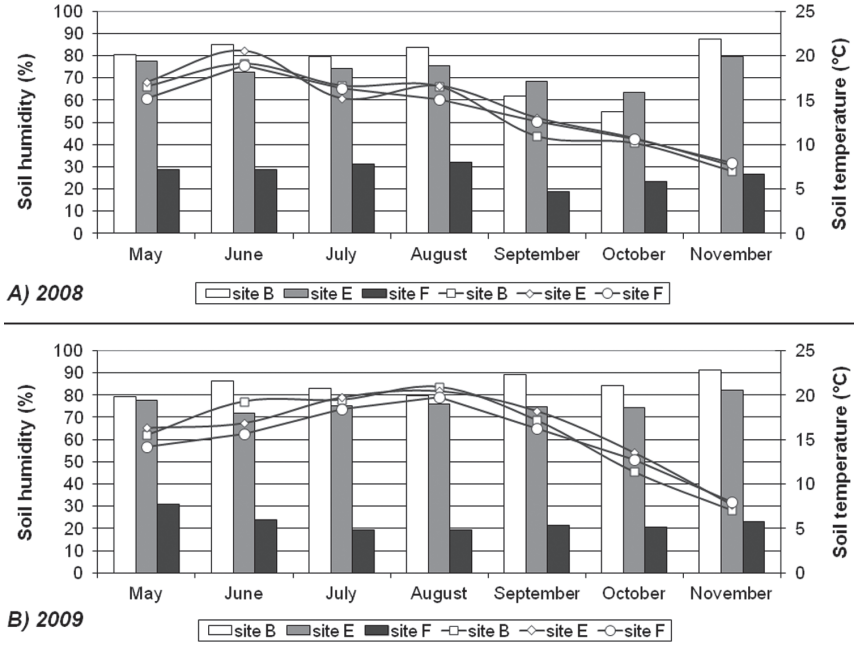

Figure 2. Soil temperature and soil humidity at the Dubravica bog and adjacent forest during 2008 and 2009. Temperature values are presented with different symbols and humidity values with bars. **Site B** – bog; **Site E** – edge; **Site F** – forest.

perature were most expressed at the edge (during both study years), especially during summer. At the bog an intermediate pattern was recorded (between edge and forest). Conversely, the lowest temperatures were measured at the bog site (during autumn and winter). Differences in soil humidity were observed in autumn between two study years, most likely due to higher amount of rain in 2009.

Data analysis

Activity-density was compared between two years, using Mann-Whiney U test and Spearman correlation. To calculate the diversity of the isopod assemblages we used Simpson (1-λ') and Shannon-Wiener indices (H'). Evenness was estimated using Pielou's evenness (J). Similarity between sites was calculated using the Bray-Curtis similarity coefficient, calculated on square root transformed and standardized abundance data (traps total catches). Non-metric MDS (multidimensional scaling) was constructed on Bray-Curtis similarity. The analyses were carried out using the PRIMER program v.6 (Clarke and Gorley 2006). Spearman's rank correlation coefficient was used to identify relationship between isopod abundance and environmental parameters (soil temperature and humidity). Activity density data did not follow normal distribution (Shapiro-Wilk test, $p<0.05$), so square root transformation was applied (Shapiro-Wilk test, $p>0.05$). ANOVA was used to compare abundances of isopods (monthly data pooled for five traps) between sampling sites. Tukey HSD (Honestly Significant Difference) test was used for *post-hoc* comparisons. Normality of data was tested using Shapiro-Wilk W test. ANOVA was executed using Statistica 7.0 (StatSoft inc.). An independent and comparable habitat fidelity index was made for collected species for each habitat type. In the case of isopods from family Oniscidae sp. and *Armadillidium* sp. habitat fidelity index was not calculated due to small number of specimens caught. It was conducted according to Nyilas (1994), based on Erdakov et al. (1979). The formula was:

$$\text{Habitat fidelity} = \frac{P_{Hi} - P_{Hi'}}{P_{Hi} + P_{Hi'}}$$

where $P_{Hi} = \frac{n_i}{n}$ and $P_{Hi'} = \frac{n - n_i}{(N-1) * n}$

n_i = number of individuals in habitat; n: number of individuals in all habitats; N: number of habitats, Hi: habitat i, Hi': all habitats except habitat i. The values of this index range from -1 to +1. The maximum value, +1 shows that all individuals of the given species were in the given habitat, whereas -1 indicates that no individuals of the species were recorded in the habitat, and 0 indicates that there was an average number of individuals in the habitat (Nyilas 1994). The sum of absolute values of the indices for a species gives the habitat selection index of the species.

Results

Species richness, activity density and diversity

During both study years, a total of 389 isopod individuals belonging to 8 species were captured (Table 2). Species richness and activity density were both lowest at the bog (over the whole study period). Overall, the highest number of individuals was recorded in the forest. ANOVA indicated significant difference in activity density between sites (d.f. = 2.42, F = 3.604, $p<0.05$). *Post-hoc* test separated activity density in the bog from values in the forest (Tukey HSD, $p<0.05$), while other combinations were not significantly different (Tukey HSD, $p>0.05$). Overall, activity density was positively correlated between the two years of the study. Moreover, no significant difference of the latter between 2008 and 2009 was recorded for site B (Mann-Whitney U-test, p=0.327), in contrast to sites E and F (Mann-Whitney U-test, p=0.013 and p=0.007). Spearman's rank correlation coefficient was statistically significant for site B (R_s=0.83, p=0.022), marginally significant for site E (R_s=0.73, p=0.10) and not significant for site F (R_s=0.55, p=0.20). Therefore, data for both years were pooled for subsequent analyses.

Soil temperature was positively correlated with the isopod total monthly catch at all three studied sites (Spearman's rank correlation; site B: N=14, R_s=0.594, p=0.025; site E: N=13, R_s=0.619, p=0.024; site F: N=14, R_s= 0.558, p=0.038). On the contrary, soil humidity negatively correlated with the isopod total monthly catch in the bog and forest samples, but the correlations were not statistically significant (Spearman's rank

Table 2. Isopod species recorded at Dubravica bog and adjacent forest with indices of diversity and evenness. **Site B** – bog; **Site E** – edge; **Site F** – forest; **Tn** – tyrphoneutral species **Tx** – tyrphoxenous species; **%** – percent share of total individuals per site.

Species name	site B	%	site E	%	site F	%	Ecological group
Armadillidium carniolense Verhoeff, 1901	8	12.7	28	19.2	3	1.7	Tn
Armadillidium sp.	0	0.0	0	0.0	1	0.5	Tn
Ligidium germanicum Verhoeff, 1901	6	9.5	3	2.1	3	1.7	Tn
Oniscidae sp.	0	0.0	2	1.4	0	0	Tn
Protracheoniscus politus C. Koch, 1841	19	30.2	61	41.8	159	88.3	Tn
Trachelipus rathkii Brandt, 1883	28	44.4	20	13.7	3	1.7	Tn
Trachelipus ratzeburgi Brandt, 1883	1	1.6	17	11.6	8	4.4	Tn
Hyloniscus adonis Verhoeff, 1927	1	1.6	15	10.3	3	1.7	Tn (possible Tx)
Number of isopod species (S)	6		7		7		
Number of isopod individuals (N)	63		146		180		
Simpson index (1-λ')	0.697		0.750		0.218		
Shannon-Wiener index (H')	1.339		1.576		0.549		
Pielou's evenness index (J')	0.748		0.810		0.283		

correlation; site B: N=14, R_s= -0.246, p=0.397; site F: N=14, R_s= -0.372, p=0.190). However, in edge samples correlations between soil humidity and the total monthly catch was positive, but also statistically not significant (Spearman's rank correlation; site E: N=13, R_s=0.077, p=0.802).

Protracheoniscus politus C. Koch was generally the most abundant species (61.4% of the total catch). Together with following species; *Trachelipus rathkii* Brandt (13.1%), *Armadillidium carniolense* Verhoeff (10%) and *Trachelipus ratzeburgi* Brandt (6.7%) it constituted 91.2% of the total catch. *T. rathkii* was the most abundant species at the bog (44.4% of the catch), while its abundance decreased at the edge, and was the lowest in the forest (reaching only 1.7%). Three following species; *P. politus* (30.2%), *A. carniolense* (12.7%) and *Ligidium germanicum* Verhoeff (9.5%) also accounted for a larger proportion of the catch at the bog. *P. politus* (41.8%) and *A. carniolense* (19.2%) were the most frequent species at the edge and *P. politus* was particularly abundant in the forest (88.3%). *Hyloniscus adonis* Verhoeff, had the highest abundance at the edge, while only 1 specimen was found at the bog. Majority of isopod taxa found at the bog are tyrphoneutral. According to diversity and evenness indices, the greatest and lowest diversity was recorded at the edge and in the forest, respectively.

Seasonal activity

Activity density of dominant *P. politus* was expressed as the total number of individuals caught monthly and plotted against time (Figure 3). The maximum seasonal activity was observed in July at both forest and edge sites. During August, the number of individuals decreased at all studied sites, whereas an increase was observed in September. Overall, low number of individuals was caught at the bog, and this was insufficient to observe seasonal dynamics. Seasonal activity density of *P. politus* did positively correlate with soil temperature at the edge and forest sites (Spearman's rank correlation; site E: N=14, R_s=0.093, p=0.751; site F: N=14, R_s=0.427, p=0.128), and negatively at the bog (Spearman's rank correlation; site B: N=14, R_s= -0.168, p=0.565). However, the correlations were not statistically significant. Additionally, Spearman's rank correlation coefficient was positive between soil humidity and seasonal activity density of *P. politus* at the bog and edge (site B: N=14, R_s=0.025, p=0.932; site E: N=14, R_s=0.033, p=0.910, respectively) and negative in the forest (site F: N=14, R_s= -0.461, p=0.096). Also, correlations were not statistically significant.

Similarity and habitat fidelity

The habitat fidelity values of collected isopod species are shown in Table 3. According to these results, two dominant species, *P. politus* and *T. rathkii,* preferred different habitat types. *P. politus* mostly inhabited forest (site F) with a high habitat fidelity value (+ 0.6), while positive habitat fidelity value was calculated for *T. rathkii* (+ 0.4) at the

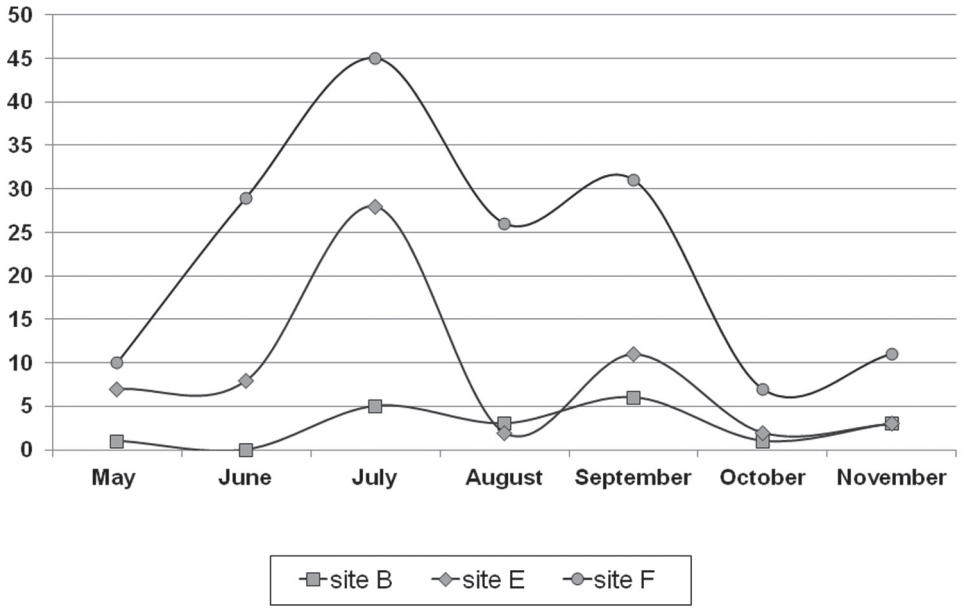

Figure 3. *Protracheoniscus politus* seasonal activity at three studied sites with different vegetation. **Site B** – bog; **Site E** – edge; **Site F** – forest; Y-axis shows total number of monthly caught individuals.

Table 3. The habitat fidelity values of dominant isopod species in each of the three habitats. **Site B** – bog; **Site E** – edge; **Site F** – forest.

	∑ N	**site B**	**site E**	**site F**	**Habitat selection index**
Protracheoniscus politus	239	- 0.7	- 0.05	+ 0.6	1.35
Trachelipus rathkii	51	+ 0.4	+ 0.1	- 0.8	1.3
Armadillidium carniolense	39	- 0.32	+ 0.67	- 0.73	1.72
Trachelipus ratzeburgi	26	- 0.88	+ 0.54	- 0.06	1.48
Hyloniscus adonis	19	- 0.8	+ 0.77	-0.47	2.04
Ligidium germanicum	12	+ 0.33	- 0.2	- 0.2	0.73

bog site. Typical hygrophylic species, *L. germanicum* and *H. adonis* also showed quite different habitat preferences. *L. germanicum* occurred mostly at the bog area (+ 0.33), while *H. adonis* preferred the edge site (+ 0.77). *A. carniolense* and *T. ratzeburgi* seemed both to prefer edge site with a higher habitat fidelity values in contrast to the values calculated for the other sites.

Non-metric MDS ordination based on Bray-Curtis similarity index, with superimposed results of cluster analysis (group-average linking), shows generally high degree of similarity between studied sites (i.e. they all cluster at 46% similarity level; Figure 4). In particular, high degree of similarity is obvious for forest and edge sites that cluster together at 71% similarity level. Forest replicate samples grouped strongly together at the same similarity level, whereas, most of the edge replicate samples clustered to-

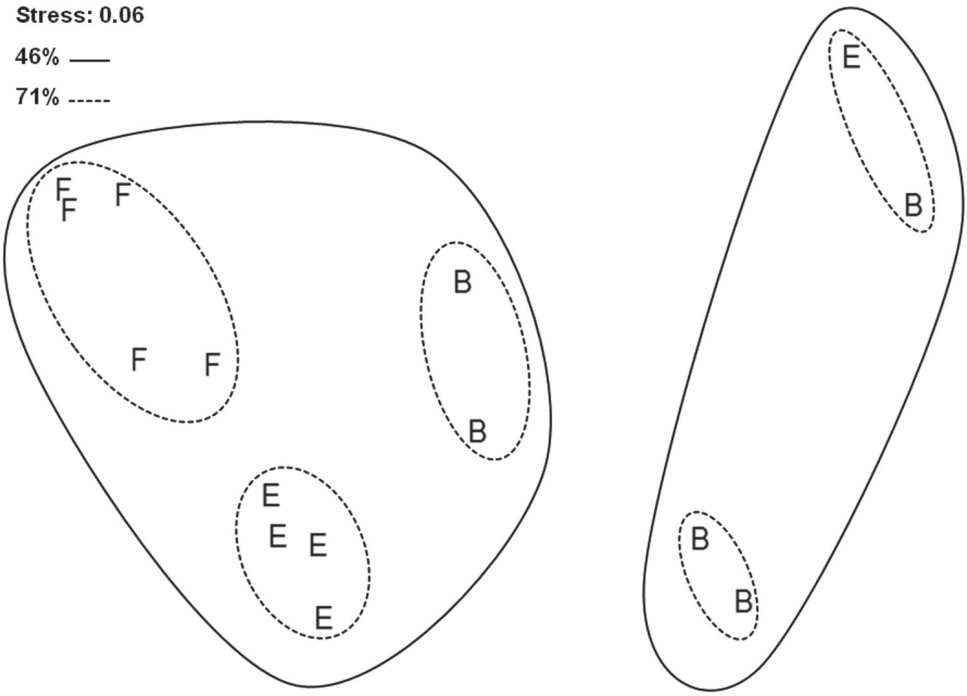

Figure 4. nMDS ordination of studied sites and Bray-Curtis similarities with superimposed results of cluster analysis. **Site B** – bog; **Site E** – edge; **Site F** – forest.

gether at 80% similarity level. On the contrary, bog replicate samples did not form a distinct group and they were combined with edge samples.

Discussion

Current study shows that isopod species richness of the isolated bog was surprisingly high and it did not considerably differ from the species richness of the edge or adjacent forest. Contrary to this, Peus (1932) found that isopods may be almost completely excluded from acidic bogs, mainly due to specific and rather extreme environmental conditions, particularly low acidity. The same was observed in isopod studies in the Canadian peat bogs (Judd 1963, Blades and Marshall 1994). According to Judd (1963) only two isopod species were recorded at the Byron bog, but none of them was found in the open bog area, than on the adjacent wooded slopes. However, our study shows completely different pattern. This is possibly a result of several factors including progressive vegetation succession, low water table and small size of the bog, thus enabling immigration from adjacent habitats. In contrast to northern acidic peat bogs where water level is at or near the surface of the substratum and the peat is infra aquatic, water level at the studied bog has considerably fallen under the minimum necessary level to prevent spreading of taller plants, such as purple moor grass (Hršak 1996).

Consequently, *Sphagnum* hummocks became less humid and such changes might have in particular affected isopod presence at the bog. Another cause might be the latitude, since generally isopod species richness increases from north to south (Hornung and Sólysmos 2007), and the same pattern was observed for e.g. spiders (Koponen et al. 2001, Koponen 2002) and ants (Gotelli and Ellison 2002) in peat bogs. Comparing our results with recent study of Farkas and Krčmar (2004) in the Danube and Drava floodplains where 11 isopod species were recorded using more pitfall traps and covering more different habitats, it seems that isopod diversity of the isolated Dubravica bog is not negligible.

The greatest diversity of isopods in the current study was found exactly in the edge mainly due to the edge effect. According to Hilty et al. (2006) ecotones are sites of high productivity and enhanced biodiversity. Furthermore, species richness of carabid beetles and ants were also highest at the edge in the Dubravica bog (Brigić et al. 2009, Bujan et al. 2010). According to the nMDS analysis, forest and edge sites show high degree of similarity most likely due their vicinity. Although these two sites differ considerably in soil humidity, it seems that forest isopod species can successfully disperse into or even inhabit the edge zone. Bog replicate samples do not form a firm group and cluster near edge sites most likely due to the process of immigration.

In our study, isopod activity density significantly differed between the bog and surrounding habitat (forest), being the lowest at the bog. This is in accordance with data from studies on other ground dwelling arthropods in peat bogs, e.g. carabid beetles (Främbs et al. 2002, Nietupski et al. 2008). Most likely, the findings are influenced by sampling method, environmental variables (such as soil humidity, pH values, vegetation structure and density) and predation pressure. Pitfall traps are most commonly used in various ecological studies of ground dwelling arthropods, such as carabid beetles (Thiele 1977, Spence and Niemelä 1994) or spiders (Uetz and Unzicker 1976). However, pitfall trapping depends on the activity of an organism (Thiele 1977), therefore factors influencing arthropod activity, also affect the number of caught individuals. Consequently, cryptozoic isopod species that live under the stones, tree bark or in the soil were under sampled or are missing from our list (e.g. (*Trichoniscus* spp., *Haplophthalmus* spp.). Furthermore, lower isopod activity might be caused by the surface structure of *Sphagnum* mosses creating a thick carpet and hence some isopod species may not be recorded. Furthermore, heavy and large bodied bumbling isopods would easily fall into the pitfall traps, whereas smaller ones would more likely be able to stop themselves on the edge (Sutton 1972).

The harsh environmental conditions, such as daily and annual temperature differences and low pH values (Figure 2; Table 1) might also have affected the isopod species richness and activity at the bog (Zimmer et al. 2000, Tuf and Jeřábková 2008). Mostly due to large calcium demands for cuticular calcification (Sutton 1972), isopods prefer to inhabit more alkaline than acid habitats (Wolters and Ekschmitt 1997). Hygropositive and photo-negative behaviour is known in isopods (Gunn 1937, Waloff 1941) enabling them to inhabit most favourable environments. Our study reveals that isopod activity (total monthly catch) was positively correlated with soil temperature.

Soil temperature fluctuates annually and daily and it is affected mainly by variations in air temperature (Zehng et al. 1993). Positive correlation could be the result of higher isopod's activity during colonisation of more suitable microhabitats (i.e. from hot and drier environments to the ones with higher humidity level and lower temperatures; Edney 1953). Similar trend was observed in correlation of isopod activity with soil humidity. Gunn (1937) and Waloff (1941) have both shown that isopod's activity has decreased at high humidity conditions. These conditions were present at the bog area, where we collected low number of isopod individuals. However, in the forest, pitfall traps were probably positioned in microhabitats with optimum ecological conditions and therefore higher numbers of individuals were collected. A comprehensive study applying additional sampling methods, such as hand collecting or extracting from soil samples with Tullgren apparatus, should provide more precise results.

Except above mentioned grounds, predation might also influence isopod activity density at the bog. High population density of lycosid spiders (Štambuk and Erben 2002) and ants (Bujan et al. 2010) were found at the studied bog. Both arthropod groups are common predators on isopods (Sutton 1970, Sunderland and Sutton 1980, Deslippe et al. 1996). According to Bujan et al. (2010) extremely high number of the ants was caught at the studied bog, precisely 13 584 individuals (while at both edge and forest sites less than 1000 individuals). Two ant species, *Myrmica rubra* L. and *M. ruginodis* Nyl., accounted for 99% of the catch. These species prefer wet habitats (Seifert 1988) and their great colony density is most likely caused by specific way of colony founding-colony budding (Steiner et al. 2005), wherefore there is one big anthill present at the bog (Bujan et al. 2010). Castillo and Kight (2005) showed that ant presence can influence both isopod behaviour (defensive and aversive behaviour) and reproductive success (shortening brooding period). Concerning the spiders, lycosid spiders were dominant members of the spider fauna at the bog and they made 72.7 % of the total catch (Štambuk and Erben 2002). According to Sutton (1970) they have a low food capacity for isopods, although in a combination of their high population density and small living area they could be considered as significant predators on isopods.

T. rathkii was the dominant species at the bog. Its abundance decreased towards the forest where the soil humidity was lower comparing to other two sites. Furthermore, its habitat fidelity value was highest at the bog (Table 3). As a widely distributed and common isopod species, *T. rathkii* inhabits different biotopes, including extreme ones (Gruner 1966), but prefers open places, and it is usually not found within woods (Schmidt 1997), which is in accordance with our study. This species is typical for flooded ecosystems (Farkas 1998, Tuf and Tufová 2005) and it is common inhabitant of wetlands and peat bogs (Potočnik 1993). Throughout the experiments, it was observed that *T. rathkii* has a high ability to survive under water and can therefore colonise such habitats (Tufová and Tuf 2005). It was also found in few other bogs in Croatia (Antonović, unpublished data). However, Judd (1963) recorded this species on the wooded slopes with no records in the bog area. However, that bog area was completely flooded and unfavourable for any terrestrial isopod species. In contrast to *T. rathkii*, *T. ratzeburgi* is a sylvan species (Farkas et al. 1999, Tuf and Tufová 2005,

Farkas and Vilisics 2008), found in relatively low numbers in all explored sites, with highest abundance at the edge.

Although species from the family Trichoniscidae, including *Hyloniscus* spp. are highly hygrophylic, and usually inhabit wetlands, species *H. adonis* is not abundant on the bog, but surprisingly on the edge. Due to bog's inclination towards the edge, high, but relatively even soil humidity was recorded throughout the sampling period at the edge site. Hence, availability of microhabitats with optimum ecological conditions for this species, but also lower predation pressure from ants and lycosid spiders could explain its highest abundance at the edge.

Among typical central European species, rather rare and less known isopod *L. germanicum* was present at all study sites. It is highly hygrophylic species (Potočnik 1993), therefore the highest abundance at the bog site is expected.

At all studied sites, *P. politus* was one of the most frequent and numerous species. It was dominant species in the forest where the soil humidity content was considerably lower. It is a xerophilous species that prefers dryer habitats (Potočnik 1993). Additionally, it is sylvan species (Karaman 1974, Tomescu et al. 2008) inhabiting woodlands, where it is a constant and dominant element (Karaman 1974, Farkas and Krčmar 2004, Tuf and Tufová 2005, Farkas and Vilisics 2008). Therefore, it is not surprising that its habitat fidelity value was highest in the forest (Table 3). *P. politus* was also found in the bog. It seems that this species can easily enter the bog area especially during the summer, most likely due to the size of the bog and progressive vegetation succession (Figure 3). The activity of *P. politus* was the highest in July and September. The soil temperatures in summer were the highest, but Spearman's rank correlation coefficient was not statistically significant, therefore soil temperature is not the key factor for activity differences. The latter is in accordance with results of Oberfrank et al. (2011). The population study from north western Romania showed also two peaks in population's dynamics, the first in April-May-June and the second in September-October (Radu and Tomescu 1972). Such seasonal activity could possibly be explained by the facultative iteroparous univoltine reproductive strategy of *P. politus*, having the reproductive season from May to August. Temporal pattern of sex ratio has its maximum (0.98 for males) by the end of April, during males-search and copulation, but the overall yearly sex ratio was around 0.27. Similar as in our study, the second reproductive peak with postmarsupial females was in July, and total female activity was highest in September (Oberfrank et al. 2011). The presence of *A. carniolense*, a further sylvan species (Tomescu et al. 2002), at the bog area is an additional evidence of strong impact of vegetation succession on the bog, changing it into a drier habitat. Similar was observed by Štambuk and Erben (2002) during the study of the lycosid fauna. Although the lycosid fauna of the bog was relatively bog-specific, changes in the habitat structure resulted in higher abundance of some forest species (Štambuk and Erben 2002).

Within the current and previous studies (e.g. Judd 1963, Blades and Marshall 1994), typical tyrphobiontic and tyrphophilous species were not recorded. On the contrary, *H. adonis* could be considered as a potential tyrphoxenous species, since it

showed some habitat preferences. Isopod fauna of peatlands is generally poorly investigated, hence further comprehensive studies providing more data on ecology of isopods inhabiting peat bogs are necessary.

Conclusions

This study reveals that vegetation succession has a strong impact on community composition of fauna inhabiting the peat bog. With overgrowth of the peat bog by *M. caerulea* grass, water level has significantly decreased. Therefore, the bog area becomes dryer, shaded and more suitable for forest species. Although there were no previous studies on isopod fauna, the presence of forest species indicates such changes in this habitat. Peat bog size and interspecific relationships, such as predation, also affected isopod species richness, activity density and diversity. Typical tyrphobiontic and tyrphophilous species were not observed, but further studies implying additional sampling methods should provide more detailed insight into isopod faunistics and ecology. In order to preserve suitable microclimatic conditions and biodiversity of the bog, management practices, like mowing, are required.

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Assemblages of terrestrial isopods (Isopoda, Oniscidea) in a fragmented forest landscape in Central Europe

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Abstract

Terrestrial isopods were collected in 13 forest fragments differing in area (within the range of 0.1 and 254.5 ha), shape and composition of forest vegetation (thermophilous oak, mesophilous oak-hornbeam, thermophilous oak-hornbeam, acidophilous oak, basiphilous oak, beech oak-hornbeam, moist mixed deciduous forest, plantations of deciduous and coniferous trees), all situated in the Český kras Protected Landscape Area, Czech Republic, Central Europe. Number of sites sampled in each fragment of forest depended on its size and ranged from 1 to 7. Altogether 30 sites were sampled. Soil samples (5 per site collected twice a year) and pitfall trapping (5 traps per site in continuous operation throughout a year) during 2008–2009 yielded a total of 14 species of terrestrial isopods. The highest densities and highest epigeic activities of terrestrial isopods were recorded in the smallest fragments of woodland. Although a wider range of habitats were sampled in the larger fragments of woodland there was not a greater diversity of species there and the population densities and epigeic activities recorded there were lower. *Porcellium collicola* was most abundant in small fragments of woodland regardless the vegetation there. *Armadillidium vulgare* and *Protracheoniscus politus* were statistically more abundant in the larger fragments of woodland. The results indicate that forest fragmentation does not necessarily result in a decrease in the species richness of the isopod assemblages in such habitats.

Keywords

Woodlice, densities, epigeic activity, pitfall trapping, *Armadillidium vulgare*, *Porcellium collicola*

Introduction

Fragmentation is a natural feature of most landscapes (Tews et al. 2004). In addition to the possible decrease in biodiversity due to loss of habitats and extinction of sensitive species (Henle et al. 2004), habitat fragmentation has weaker positive than negative effects on biodiversity (Fahrig 2003). Invertebrates may react to habitat fragmentation differently (Ewers and Didham 2006) as it can positively influence the development and diversification of habitats and more-specialized species may be more susceptible to these processes than generalists (Didham et al. 1996).

The Central European landscape has been influenced by human activities for millennia. It is characterised by intensive land-use and a continuing trend of habitat destruction. Originally the landscape in the temperate zone was covered with woodland. Present forest system consists of patches differing in shape, size and vegetation in which there is often a higher biodiversity than in the majority of open and usually agriculturally exploited areas. Fragmentation of remaining areas of natural, semi-natural and other well-preserved patches of forest represents a major threat to biodiversity (Tscharntke et al. 2002). However, it is unknown whether such processes also influence the diversity of soil invertebrates (Didham et al. 1996). Although some studies suggest that soil organisms, in general, are not sensitive to habitat fragmentation, even at a small scale (Rantalainen et al. 2008, David and Handa 2010), this is not the case for rare, more specialised species or those with a poor dispersal ability (Tscharntke et al. 2002).

In the assessment of the effect of forest fragmentation on vegetation and different groups of aboveground and soil invertebrates, terrestrial isopods are used as a model group of soil saprophagous macro-invertebrates. Terrestrial isopods are also potential bio-indicators of environmental quality in natural as well as disturbed and polluted habitats (Dallinger et al. 1992, Paoletti and Hassall 1999). Presented in this paper is a comparison of the assemblages of terrestrial isopods in forest fragments differing in area and other structural parameters. The aim of the paper is to assess how the above (epigeic activity) and belowground (soil) parts of assemblages of terrestrial isopods react to forest fragmentation.

Materials and methods

This research was undertaken in the fragmented landscape of the Český kras Protected Landscape Area, Central Bohemia, Czech Republic. In this area the bedrock is predominantly limestone and there are numerous fragments of formerly more integrated woodlands. There is little diversity in the structure of the vegetation in the smaller fragments, which contrasts with the mosaic character and higher spectrum of plant associations with a long history of diverse management and development in the larger forest units. During the period 2008–2009 soil sampling and pitfall trapping were used to determine the assemblages of terrestrial isopods in 13 fragments of forest that ranged in area between 0.1 and 254 ha. A total of 30 sites were sampled (Table 1). The

Table 1. The species of terrestrial isopods present at the different sites studied recorded in (▲) pitfall trap catches, (▼) extracts of soil samples and (+) by both methods. Total number of each species collected at each site in each fragment of woodland, frequency of occurrence of individual species (F) in % and abbreviations of species names used in the statistical analyses.

Fragment of woodland (ha)	0.1	0.3	0.8	4.5	9.5	11.2		13.6		14.2		15.3	18.9				20.9					35.9		254.5							
Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
Vegetation	BO	TO	DP	TO	MOH	DP	AO	MOH	MDF	AO	AO	MOH	TOH	TOH	MOH	DP	MOH	BO	CP	TO	BO	TOH	TOH	CP	TO	BOH	TO	TO	AO	MOH	F (%)
Armadillidium vulgare (Latreille, 1804) Armvul	+	+	+	+	+	+	+	+	▲	+	+	+	+	+	+	+	▲	+	+	+	+	+	+	+	+	▲	+	+	+	+	100.0
Cylisticus convexus (De Geer, 1778) Cylcon	▲	-	-	-	-	▲	▲	-	-	-	-	-	-	-	-	-	-	-	▲	-	-	-	-	▲	-	-	-	-	-	-	16.7
Haplophthalmus mengii (Zaddach, 1844) Hapmen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	3.3
Hyloniscus riparius (C.L.Koch, 1838) Hylrip	-	-	▲	-	-	▲	▲	▲	▲	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.7
Lepidoniscus minutus (C.L.Koch,1838) Lepmin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	▲	-	-	-	-	3.3
Platyarthrus hoffmannseggii Brandt, 1833 Plahof	-	-	▲	-	-	▲	▲	-	-	-	-	-	-	▲	▼	-	-	▲	-	-	-	-	-	-	-	-	-	-	-	-	20.0
Porcellio scaber Latreillle, 1804 Porsca	-	-	-	-	-	▲	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3
Porcellionides pruinosus (Brandt, 1833) Porpru	▲	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3
Porcellium collicola (Verhoeff, 1907) Porcol	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	▲	▲	▲	▲	+	▲	-	+	▲	-	+		+	+	▼	86.7
Protracheoniscus politus (C.Koch, 1841) Propol	+	+	-	+	+	-	-	+	-	+	+	+	+	▲	+	+	+	▲	+	+	+	+	+	+	+	+	+	+	+	+	86.7
Trachelipus nodulosus (C.Koch, 1838) Tranod	-	-	-	-	-	-	▲	-	-	-	-	-	-	-	▲	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7

Fragment of woodland (ha)	0.1	0.3	0.8	4.5	9.5	11.2		13.6		14.2		15.3	18.9					20.9					35.9		254.5							
Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
Vegetation	BO	TO	DP	TO	MOH	DP	AO	MOH	MDF	AO	AO	MOH	TOH	TOH	MOH	DP	MOH	BO	CP	TO	BO	TOH	TOH	CP	TO	BOH	TO	TO	AO	MOH	F (%)
Trachelipus rathkii (Brandt, 1833) Trarat	-	▲	-	-	-	▲	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7
Trachelipus ratzeburgii (Brandt, 1833) Traraz	-	-	▲	+	+	▲	-	-	▲	▲	▲	▲	▲	-	▲	▲	▲	-	+	+	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	80.0
Trichoniscus pusillus Brandt, 1833 Tripus	-	-	-	-	-	-	-	▼	+	-	-	-	-	-	-	-	-	▼	-	-	-	-	-	-	-	▲	▲	-	-	-	16.7
Total number of spp.	5	4	5	4	4	8	6	5	5	4	4	3	4	4	6	4	4	5	5	4	4	3	4	5	3	7	4	4	4	4	
Species per fragment	5	4	5	4	4	9		8		4		3	6				7					4		8							

sites sampled were representative of the seven forest plant associations characteristic of the area: thermophilous oak (TO), mesophilous oak-hornbeam (MOH), thermophilous oak-hornbeam (TOH), acidophilous oak (AO), basiphilous oak (BO), beech oak-hornbeam (BOH), moist mixed deciduous forest (MDF) and plantations of deciduous (DP) and coniferous (CP) trees. At each site, five soil samples (area of each 625 cm^2, depth ca 10 cm) were collected in spring and autumn and isopods were heat extracted using a modified Kempson apparatus (Kempson et al. 1963). In addition, five pitfall traps (each with a diameter of 9 cm and containing a solution of formaldehyde) were used to catch isopods, which were collected from the traps once a month for a period of year at each study site. Additional soil samples were collected at each site and used to determine the chemical characteristics of the uppermost soil layers (soil pH, C:N ratio and Ca^{2+} content).

The data on the isopod assemblages were evaluated with respect to the parameters of the sites and fragments. Multivariate analyses: DCA and RDA were used to consider gradient data length and importance of factors shaping the terrestrial isopod assemblages (13 variables). Methods of material collection (pitfall traps and soil samples) were treated as covariables in block analysis. The importance of the explanatory variables considered was examined during forward selection procedure in RDA. The analyses were done using the software for canonical community ordination – Canoco 4.5/CanoDraw 4.14 (ter Braak, Šmilauer 2002).

Results

During the course of the two years of this study we collected 28 thousand isopods (1,839 were extracted from soil samples and 26,398 were caught in the pitfall traps) belonging to 14 species (Table 1). The most abundant of which were *Armadillidium vulgare*, *Porcellium collicola* and *Protracheoniscus politus*. The first species was recorded at all the sites and the frequency of occurrence of the other two species was 86.7 %. High frequency of occurrence (80.0 %) was recorded also for *Trachelipus ratzeburgii*. Whereas the first three species were generally predominant in both the pitfall trap catches and soil extracts *Trachelipus ratzeburgii* mainly inhabits aboveground microhabitats and is frequently found under the bark of decaying wood. Thus, it was repeatedly caught in pitfall traps, but often absent in extracts of soil samples.

Individual sites were frequently inhabited by only four or five species. The highest number of species was found in both small and large fragments (9 and 8 species in fragments of 11.2 and 254.5 ha, respectively).

Even the smallest fragments, which varied little in the structure of their vegetation, harboured high population densities of a high number of species. Both, the highest population density (549 individuals per m^2) and epigeic activity (4,206 individuals per 5 traps per year) were recorded in the smallest fragments (Figure 1 and 2). In spite of sampling a greater number of sites in the medium-sized and large fragments of forest the lowest population densities and epigeic activities were recorded there.

Figure 1. Total population densities (ind.m-2 ± SE) (columns) and numbers of species (white squares) in the isopod assemblages at the sites sampled in the different fragments of woodland.

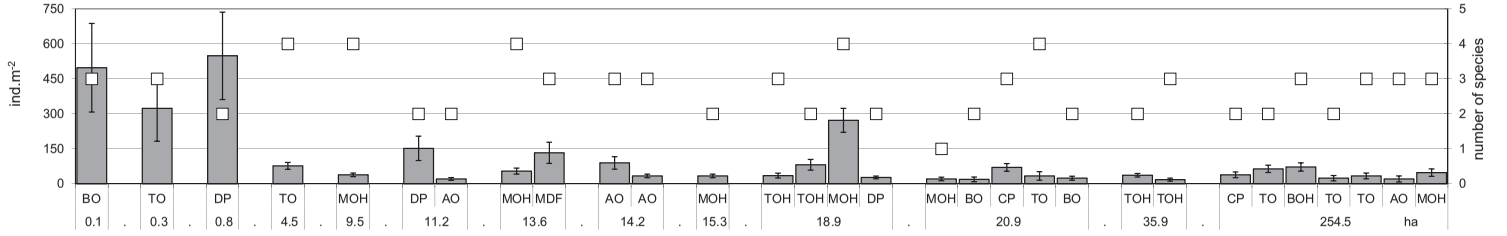
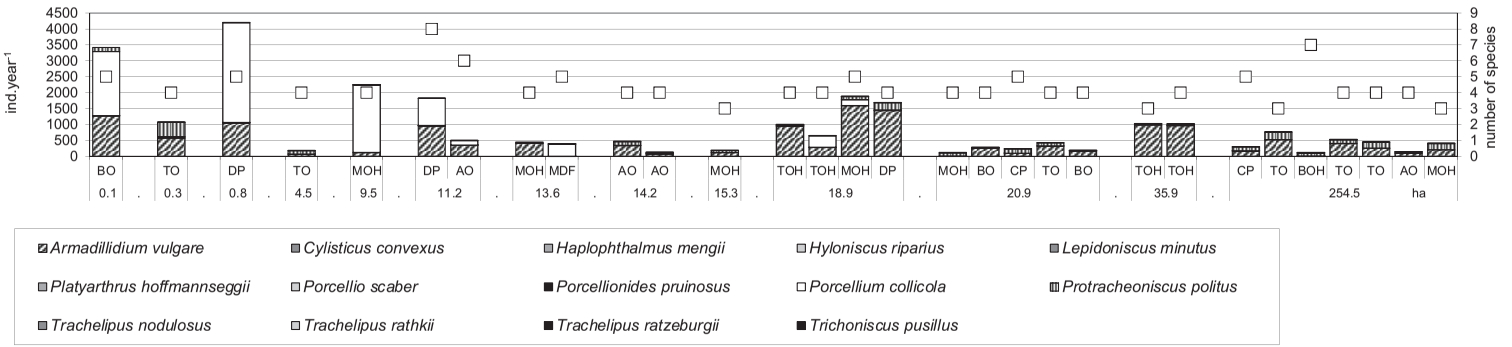


Figure 2. Epigeic activity (columns) (total catch per 5 traps per year) and numbers of species (white squares) of isopods at the sites studied in the different fragments of woodland.



Since the weighted-average Detrended Correspondence Analysis (DCA) indicates that isopods have a rather low beta-diversity, i.e. a 'short gradient' (=2.265), where most species show a linear response to the explanatory variables considered (Leps and Smilauer 2003), a triplot of the Redundancy Analysis (RDA) is presented (Figure 3). This indicates

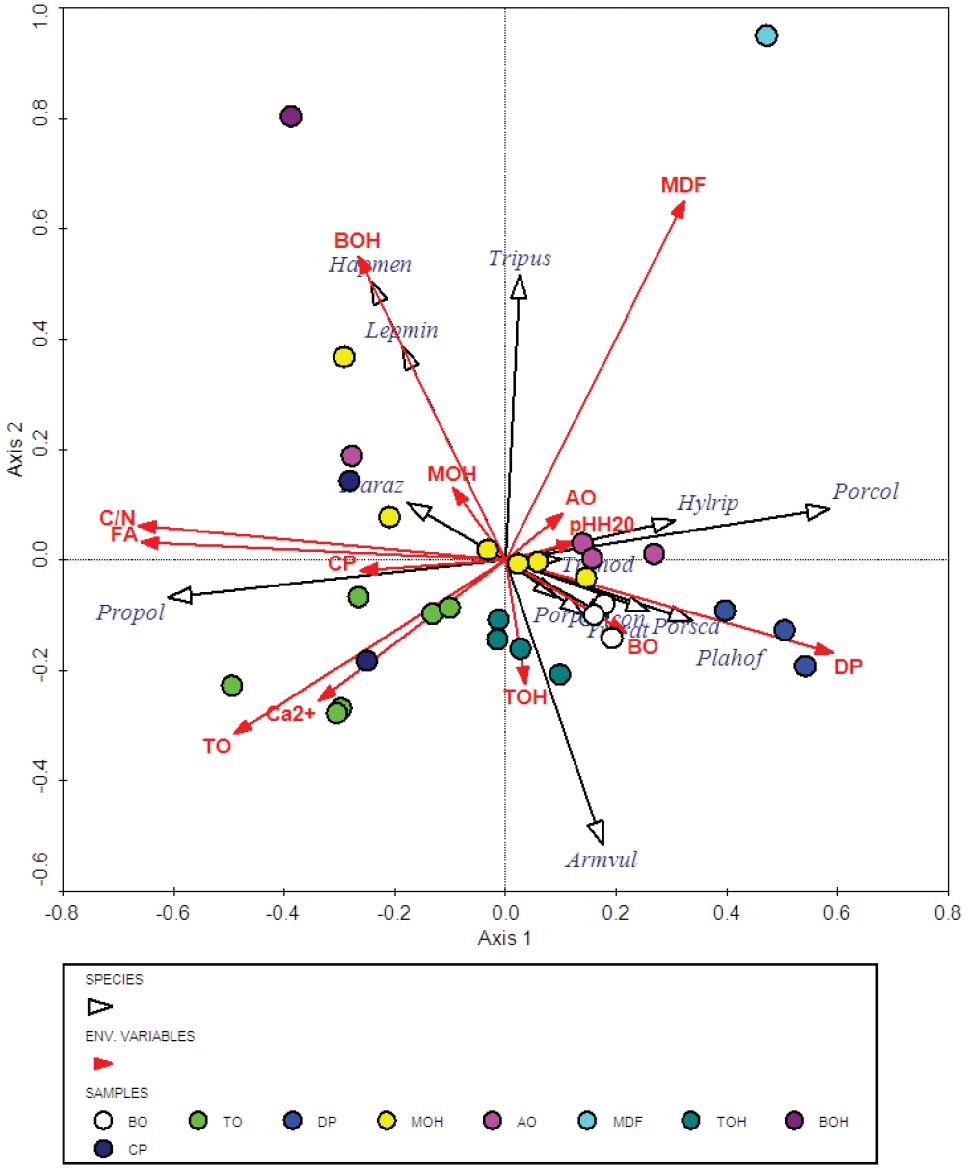


Figure 3. A triplot of the RDA block analyses of terrestrial isopod assemblages at the sites studied. For the abbreviations of the species see Table 1, FA – fragment area, C/N – carbon-nitrogen ratio, Ca2+ – calcium content of the soil, pHH2O – soil acidity. TO, MOH, TOH, AO, BO, BOH, MDF, DP and CP – vegetation at the different sites, see text.

that the 1st canonical axis is correlated negatively mainly with the area of the fragments (FA) and C:N ratio (Figure 3). Most species are significantly positively correlated with the 1st axis. *Protracheoniscus politus* is correlated positively with size of a fragment and the C:N ratio whereas the second most abundant species, *Porcellium collicola,* is negatively correlated with these variables. The abundance of *Armadillidium vulgare*, which was abundant at most sites, was not associated with either FA or the C:N ratio. The two relatively rare species *Haplophthalmus mengii* and *Lepidoniscus minutus* were mainly associated with beech oak-hornbeam sites (BOH) and low population densities of the hygrophilous *Trichoniscus pusillus* and *Hyloniscus riparius* partly with moist deciduous forest.

The forward selection procedure revealed that the area of a fragment was the most important significant variable (marginal effect: λ1 = 0.1) determining the assemblage of terrestrial isopods (Table 2). The C:N ratio was highly correlated with FA and also may influence the variability in terrestrial isopod assemblages (marginal effect: λ1 = 0.1), however this association is not statistically significant.

Table 2. The results of the verification of the explanatory variables using the Monte Carlo permutation test (499 permutations under reduced model) in the forward selection procedure of RDA (CANOCO 4.5); significant variables are in bold.

Marginal Effects			**Conditional Effects**				
Variable	Var.N	Lambda1	Variable	Var.N	LambdaA	P	F
FA	**4**	**0.10**	**FA**	**4**	**0.10**	**0.002**	**8.51**
C:N	3	0.10	**MDF**	**10**	**0.06**	**0.002**	**4.94**
DP	**7**	**0.08**	**DP**	**7**	**0.05**	**0.008**	**5.53**
TO	6	0.07	**BOH**	**13**	**0.04**	**0.020**	**3.60**
MDF	**10**	**0.06**	TO	6	0.02	0.076	2.45
BOH	**13**	**0.05**	MOH	8	0.01	0.264	1.22
Ca2+	2	0.03	CP	12	0.02	0.192	1.48
CP	12	0.02	TOH	11	0	0.440	0.83
BO	5	0.02	C/N	3	0.01	0.398	1.00
TOH	11	0.01	BO	5	0.02	0.144	1.82
MOH	8	0.01	Ca2+	2	0.02	0.158	1.65
pHH_20	1	0.01	pHH_20	1	0	0.962	0.17
AO	9	0					

Discussion

The three predominant species are differently associated with the area (FA), C:N ratio, soil acidity (pH) and Ca2+ content of the soil in the fragments of woodland studied. The isopods *Armadillidium vulgare* and *Porcellium collicola* were more abundant in small fragments of woodland. Nevertheless, *Armadillidium vulgare* was frequently recorded in the largest fragments. *Protracheoniscus politus* was most abundant in large fragments of woodland. The RDA also reveals a close association of *Armadillidium vulgare* and *Porcellium collicola* with fragments of woodland with base rich soils (high pH), whereas for *Protracheoniscus politus* this association is less pronounced. Some spe-

cies (e.g. *Cylisticus convexus*, *Trachelipus ratzeburgii*, *Porcellio scaber*, *Trachelipus nodulosus* and *Trachelipus rathkii*) may be more acid tolerant.

Although *Armadillidium vulgare* was abundant at most of the sites studied, both in small and large fragments of woodland (see Figure 2), *Porcellium collicola* was most abundant in the small fragments of woodland irrespective of the vegetation at these sites. The third most frequent species, *Protracheoniscus politus*, was most closely associated with large fragments of woodland. Its occurrence in some of the smaller fragments may be attributed to the historical fact that mainly due to man the forest in this area was fragmented into small separate wooded islands during the course of the past century.

The isopod communities in the larger fragments of forest, which have the greatest diversity of habitats, were the most homogeneous. Surrounding open grassland and forest-steppe calcareous biotopes, including diverse man-made habitats, did not enrich the diversity of isopods recorded in forest fragments as synanthropic species were always in the minority.

The abundance and composition of the species in isopod assemblages differed depending on the plant associations in the fragments of woodland sampled, but in vegetation types TO, TOH and AO they were very similar.

Terrestrial isopods do not appear to be more sensitive to fragmentation than other saprophagous invertebrates, such as millipedes (David and Handa 2010). The fragmentation of shrubby habitats in urban areas does not reduce the epigeic activity of *Armadillidium vulgare* and *Porcellio laevis* (Bolger et al. 2000). Apparently the critical fragment size for these animals is very small or they are better at dispersing than generally thought (David and Handa 2010).

Our results indicate that forest fragmentation does not necessarily result in a decrease in the size of terrestrial isopod assemblages, but their dominance structure may be affected, which is in accordance with the results presented in the David and Handa's (2010) review. The determination of the sensitivity of different species to fragmentation is dependent on further analyses of the changes in the population parameters associated with other environmental characteristics that occur following fragmentation.

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Occurrence and assemblage composition of millipedes (Myriapoda, Diplopoda) and terrestrial isopods (Crustacea, Isopoda, Oniscidea) in urban areas of Switzerland

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Abstract

Terrestrial isopods and millipedes, members of the invertebrate macro-decomposer guild, were collected through pitfall traps in three Swiss cities (Zurich, Lucerne, Lugano). A total of 7,198 individuals of 17 isopod species (7093 ind.), and 10 millipede species (105 ind.) were captured. Besides the Alpine endemic isopod *(Trichoniscus alemannicus*) and millipede *(Cylindroiulus verhoeffi)*, urban assemblages were mainly composed of widespread, native European and even cosmopolitan species, which are frequent in anthropogenic areas. Overall species richness (isopods and millipedes combined) was similar in Zurich (17 species) and Lucerne (16), while only 13 species were sampled in Lugano. According to the Sørensen index of similarity, species composition of Zurich and Lucerne were more alike, while the one of Lugano was more distinct from the other two cities. This result can be explained by the spatial proximity of Zurich and Lucerne in the north of the Alps compared to Lugano, which is located more distantly and in the south of the Alps. Dominant isopods and millipedes in Zurich and Lucerne were found to be widespread synanthropic species in temperate Europe (*Porcellio scaber, Trachelipus rathkii* and *Ophyiulus pilosus*) while the dominant isopod in Lugano (*Trachelipus razzautii*) is a species with a north-eastern Mediterranean distribution. Our study reveals that the urban millipede and isopod fauna in Swiss cities mainly consists of widespread species, but species of narrower distribution (e.g. *T. alemannicus*, *C. verhoeffi*) may also find suitable habitats in cities. Despite some signs of biotic homogenization, our study also found compositional differences of millipede and isopod assemblages between northern and southern cities that suggest geographical effects of the regional species pool.

Keywords
Decomposers, urbanization, woodlice, urban biodiversity, arthropods

Introduction

As one of the major factors of global change, urbanization and its effects on biodiversity have attracted great scientific attention in the past decade (e.g. McDonnell and Hahs 2008, Niemelä et al. 2000). Numerous studies have added an increasing knowledge to the understanding of the ecology of many taxa in urban environments, such as plants (e.g. Walker et al. 2009, Gulezian and Nyberg 2010), insects (e.g. Magura et al. 2004, Sattler et al. 2010a, 2010b, 2011), spiders (e.g. Magura et al. 2010), and vertebrates (e.g. Melles et al. 2003, Tóth et al. 2009, Fontana et al. 2011).

Urban soil meso- and macro-arthropods have received less attention (but see Korsós et al. 2002, Vilisics et al. 2007), despite their importance in ecosystem processes such as decomposition of organic matter (Hassall et al. 1987). Decay of dead plant matter results in ions readily available to uptake for plants. The majority of fallen leaves and woody debris are broken down by microbes, fungi and invertebrates (Berg and McClaugherty 2003).

Soil macro- meso- and micro-invertebrates contribute in the decomposition cascade by either fragmenting, or further mineralizing dead plant matter (Verhoef and Brussaard 1990). Key organisms in initial breakdown and comminution of dead matter are isopods, millipedes, termites, ants, and members of other invertebrate groups (Coleman and Hendrix 2000), while nematodes and annelids are essential for mineralization (Verhoef and Brussaard 1990, Flegel and Schrader 2000). Thus, millipedes (Myriapoda: Diplopoda) and isopods (Crustacea, Isopoda: Oniscidea) belong to the same functional guild, even though they are taxonomically quite distant (Scheu and Falca 2000). Based on this common ecosystem function, we propose to pool the two taxa in the same analyses which, to the best of our knowledge, is a rather novel approach in soil zoology and ecology.

Millipedes and isopods are known to inhabit European urban habitats, mainly by cosmopolitan and Holarctic species (Smith et al. 2006, Riedel et al. 2009, Vilisics and Hornung 2009). Human activities, such as gardening, transportation of soil, and cultivation of ornamental plants, are suspected to be the most important factors for species exchanges of less mobile organisms such as millipedes and isopods between distant locations. At the same time other studies (Bogyó and Korsós 2009, Riedel et al. 2009) have shown that also the native species pool has an effect on urban soil fauna, providing substrate for native species typical in natural and semi-natural habitats in cities. Particular urban habitats, such as botanical and private gardens and parks, however, harbour established populations of alien soil arthropods too (Vilisics and Hornung 2009, Cochard et al. 2010).

Urban assemblages of soil invertebrates show controversial patterns of species composition: some studies suggest that species compositions differ along urbanization gra-

dients (Korsós 1992, Korsós et al. 2002, Bogyó and Korsós 2009, Riedel et al. 2009), while others have shown no differences along similar gradients (Hornung et al. 2007, Vilisics et al. 2007). In this study we analyse the occurrence and assemblage composition of urban millipede and isopod faunas in three urban areas of Switzerland. We discuss the most interesting findings with respect to European soil fauna. This contribution is part of the BiodiverCity project (www.biodivercity.ch) that aims to assess biodiversity in urban environments and its acceptance by citizens in the framework of the Swiss national research program 'Sustainable development of the built environment'.

Methods

Study sites

The study took place in three Swiss cities, namely Zurich (371,000 inhabitants /92 km^2), Lucerne (59,000 /24 km^2) and Lugano (49,000/ 26 km^2), which represent small to medium sized cities in central Europe. The cities studied lay on a north to south gradient (approx. 200 km, with Lugano south of the Alps) and all are bordered by a lake and mountains > 800 m. Originally, 36 sampling sites were selected in each city but at the end only 106 could be used for the analyses: 36 in Zurich and Lugano, 34 in Lucerne.

The three cities share common features such as historical centres, residential areas, business quarters, public green areas, parks and cemeteries, and former industrial areas. The cities are characterized by moderate temperature (North: average January temperature 1°C, July 17°C; South: January 3°C, July 20°C) with an annual precipitation of 1000 mm for Zurich, 1150 mm for Lucerne and 1600 mm for Lugano.

Within each of the three cities sampling points were selected along a continuous urbanization gradient, which was measured as the fraction of sealed and built area in the 50 m radius around the sampling points. The selection of the individual sampling points followed a reasoned choice sampling strategy to cover the entire urbanisation gradient (3% to 92% sealed and built area). We included a wide range of urban habitat types (private gardens, semi-public spaces of apartment buildings, public parks and courtyards of industrial buildings) into the study. Mean distance between study sites was 388 m (± 21 m SE). A minimal distance of 250 metres was kept between sampling sites and the city fringe. Precise locations of the study sites are given in Germann et al. (2008).

Data collection

Isopods and millipedes were sampled through pitfall traps, consisting of 3 plastic cups (opening diameter 75 mm) per trap site recessed into the soil and arranged in an isosceles triangle with a distance of one meter. Transparent roofs installed approximately 8 cm above the cups provided protection from rain. Traps were emptied weekly during

7 weeks from June 13th to August 3rd 2006 (Sattler et al. 2010a), which corresponds to the period with highest arthropod activity in Switzerland (Duelli et al. 1999, Obrist and Duelli 2010).

Identification of millipedes was based on Schubart (1934) and Blower (1985). For isopod identification we used the keys of Schmölzer (1965) and Gruner (1966). Valid nomenclature was applied according to Schmalfuss (2003) and Enghoff (2010). The reference collection is deposited at the Natural History Museum in Lugano (Switzerland).

Data analyses

We used species richness (number of species) as the most common measure to quantify biodiversity (Magurran 2004). Incidence is the frequency with which the species occurs at all in the study sites of a city. This value was used to assess steadiness of a species in the three cities. This value is indicative to the regularity of a species' occurrence which does not necessarily correlate with abundance. We regarded incidence rate 'high' when it was over 50%, and 'low' when it did not reach 10%.

The similarity between the millipede and isopod species assemblages combined sampled in the three cities was assessed using the Sørensen index (Sørensen 1948), which is a widely used index in ecology and thus suitable for comparative purposes. For these analyses we used data of specimens identified to species level only.

Results

Species richness and composition

Overall, 17 species of isopods (7015 individuals) and 8 species millipedes (98 ind.) were identified in the three studied Swiss cities; one isopod could only be identified at genus level, while two millipedes only at family level.

Isopod species richness was highest in Lucerne (14 species) and lowest in Lugano (10 species), with 11 species in Zurich (Table 1). One third (6 species) of all Isopoda species occurred in all three cities. Five additional species were captured in both Lucerne and Zurich but not in Lugano (Table 1). Three isopod species were dominant in the three cities (Fig. 1), i.e. the cosmopolitan *Porcellio scaber* Latreille, 1804 in Zurich (1216 individuals, 32% relative abundance); the widespread European *Trachelipus rathkii* (Brandt, 1833) in Zurich (1934, 33%) and Lucerne (1234, 72%), and the Mediterranean *Trachelipus razzautii* (Arcangeli, 1913) in Lugano (307, 52.3%).

We found seven millipede species in Zurich, four in Lugano, and three in Lucerne. *Ophyiulus pilosus* (Newport, 1842) occurred in all three cities, but was dominant in Zurich (62.5%) and Lugano (42.5%), while *Polydesmus angustus* Latzel, 1884 was dominant in Lucerne (85%) (Fig. 1). Two other species [*Brachydesmus superus* Latzel, 1884 and *Oxydus gracilis* (C. L. Koch, 1847)] were exclusively found in Zurich and Lucerne (Table 1).

Table 1. Incidence of isopod and millipede species in sampling sites in the cities of Zurich, Lucerne, and Lugano.

Species	Species occurrences in traps		
	Zurich (n=36)	**Lucerne (n=34)**	**Lugano (n=36)**
Androniscus dentiger Verhoeff, 1908	+	+	0
Armadillidium nasatum Budde-Lund, 1885	+	+	+
Armadillidium vulgare (Latreille, 1804)	+	+	+
Cylisticus convexus (De Geer, 1778)	+	+	0
Haplophthalmus danicus Budde-Lund, 1880	0	0	+
Hyloniscus riparius (C. Koch, 1838)	+!	+!	+
Ligidium hypnorum (Cuvier, 1792)	0	+	0
Oniscus asellus Linnaeus, 1758	+!	+!	0
Orthometopon planum (Budde-Lund, 1885)	0	0	+
Philoscia muscorum (Scopoli, 1763)	+	+!	0
Platyarthrus hoffmannseggii (Budde-Lund, 1893)*	+	+	+
Porcellio scaber Latreille, 1704	+!	+!	+
Porcellionides pruinosus (Brandt, 1833)	+	0	0
Trachelipus rathkii (Brandt, 1833)	+!	+!	+
Trachelipus razzautii (Arcangeli, 1913)	0	0	+!
Trichoniscus alemannicus Verhoeff, 1917	+	+	0
Trichoniscus pusillus Brandt, 1833	0	+	0
Trichoniscus sp.	0	+	+
Isopod species	**11**	**13**	**9**
Isopod specimens	**3738**	**2690**	**587**
Brachydesmus superus Latzel, 1884	+	0	+
Cylindroiulus caeruleocinctus (Wood, 1864)	+	0	0
Cylindroiulus verhoeffi (Brolemann, 1896)	0	0	+
Nemasoma varicorne C. L. Koch, 1847	+	0	0
Ophyiulus pilosus (Newport, 1842)	+	+	+!
Oxidus gracilis (C. L. Koch, 1847)	0	+	+
Polydesmus angustus Latzel, 1884	0	+	0
Propolydesmus testaceus (C. L. Koch, 1847)	+	0	0
Chordeumatidae sp.	0	+	0
Craspedosomatidae sp.	0	0	+
indet +	+	0	0
Millipede species	**5**	**3**	**4**
Millipede specimens	**32**	**27**	**46**

Legend: 0: species absent; +: species present; +!: species present at ≥ 25% of sites per city; * taxa identified as a myrmecophilous species (ubiquitous in ant nests); number of specimens were not counted, see text for explanation

Millipedes show a different occurrence pattern than isopods, for the latter the differences among the three cities seem to be greater. Overall, 50% of all millipede and isopod species were observed in only one city. The mean number of individuals

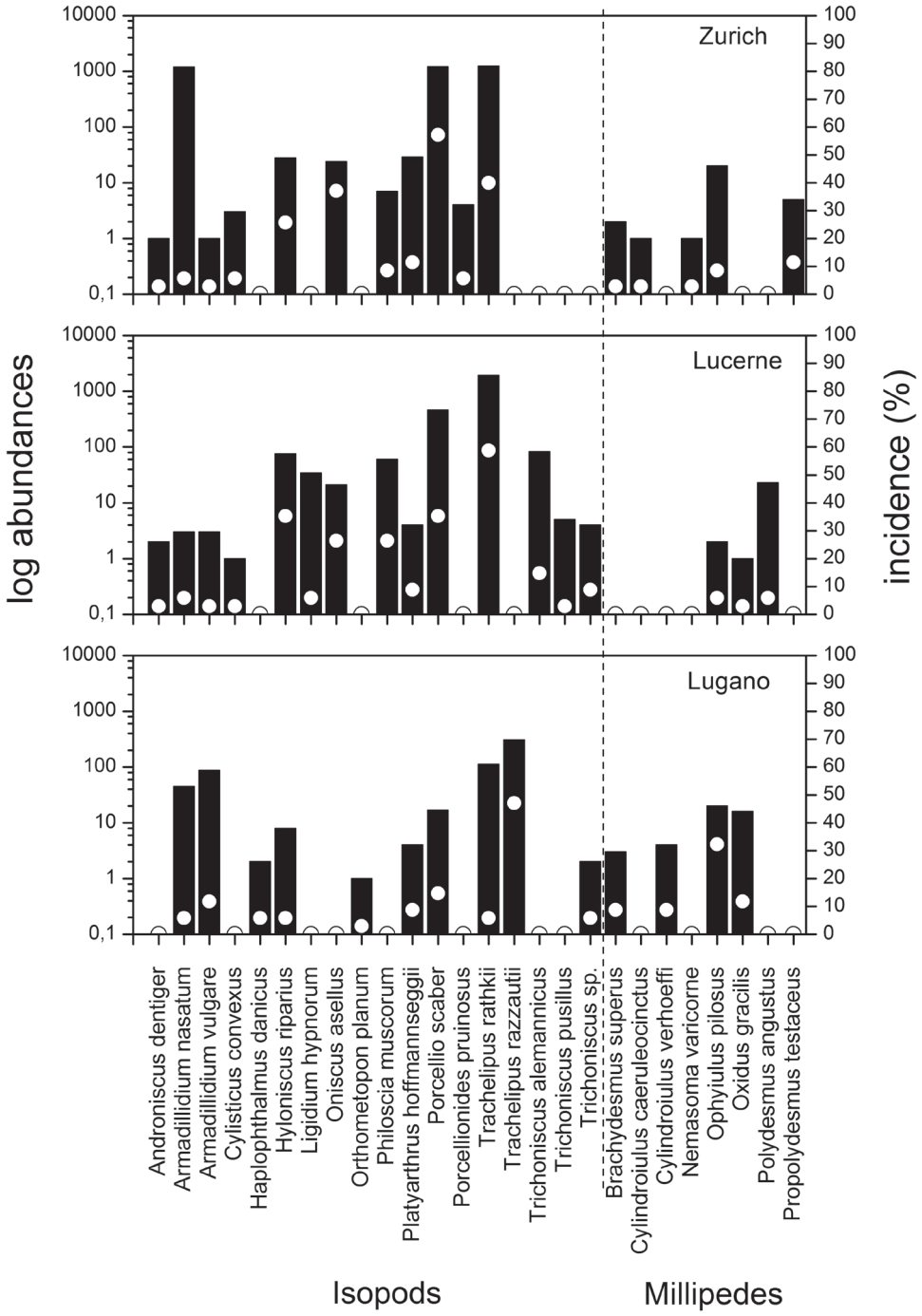

Figure 1. Abundances of isopods and millipedes in Zurich, Lucerne and Lugano in logarithmic scale. Dashed line separates isopods (on the left) from millipedes. Black bars represent abundances, white circles show the species' incidences per all sites per city.

for isopods and millipedes per site varied substantially in the three cities, i.e. Lucerne [mean 159.7 (SE: 75.3)], Lugano [mean 45 (SE: 12.56)], and Zurich [mean 221.7 (SE: 77.22)]. Sørensen similarity index of species compositions (isopods and millipedes combined) was highest between Zurich and Lucerne (0.67) and lowest between Zurich and Lugano (0.40), with an intermediate value of 0.58 between Lucerne and Lugano.

Species incidence

Incidences of isopod and millipede species per city were generally low, i.e. below 10% of the total number of traps (Figure 1). For isopods, the most abundant species were also the most frequent ones. The most widespread isopod in Zurich was *P. scaber* (55.6% of 36 traps), while in Lucerne it was *T. rathkii* (58.8% of 34 traps), and in Lugano *T. razzautii* (44.4% of 36 traps). Lucerne and Zurich, cities located north of the Alps, shared four isopod species [*Hyloniscus riparius* (C. Koch, 1838), *P. scaber*, *Oniscus asellus* Linnaeus, 1758, and *T. rathkii*] out of the five most frequently sampled (≥ 25%). The only millipede with an incidence over 25% (out of 36 traps) was *O. pilosus* in Lugano, while the rest of millipede species of our study occurred with incidences < 10% (Figure 1).

There were also examples where higher relative abundances paired with relatively low incidences (<10% occurrence per all sites or per all city) which suggest an aggregated distribution of some species. This was the case for *T. rathkii* (relative abundance 20%; incidence 6%) and *Armadillidium vulgare* (Latreille, 1804) in Lugano (14%; 6%), and *Armadillidium nasatum* Budde-Lund, 1885 in Zurich (30%; 6%).

Discussion

Faunistic results

Next to many taxonomic and faunistic studies on European isopods (Schmalfuss and Wolf-Schwenninger 2002), to the best of our knowledge, there were neither reviews nor faunistic papers published on Swiss Isopoda fauna in the last 100 years since the last overview by Carl (1911). The recent millipede fauna has been summarized by Pedroli-Christen (1993), describing 127 species from the country. Therefore our results are somewhat challenging for interpretation and need to be put in context by considering other studies on the European level.

Our results reveal that the observed cities harbour mostly species widespread in Europe. Regarding isopods, six species are known as widespread in temperate and northern Europe, occupying both urbanized and rural areas: *A. vulgare, O. asellus, Philoscia muscorum* (Scopoli, 1763), *P. scaber, T. rathkii, Trichoniscus pusillus* Brandt, 1833 (e.g. Hornung et al. 2007, Vilisics et al. 2007). *Orthometopon planum* (Budde-Lund, 1885), a Central European species, is known to dwell in broadleaf forests

(Vilisics et al. 2008), but has also been found in the urban fringe of Budapest (Vilisics and Hornung 2009). The only frequent isopod species in Lugano, *T. razzautii*, seems, instead, to be mainly restricted to northeastern Mediterranean (Schmalfuss 2003).

Among millipedes, seven species are widely distributed across Europe (Enghoff 2010), six of which are known to occur in areas under human influence: *B. superus*, *Cylindroiulus caeruleocinctus* (Wood, 1864), *O. pilosus*, *O. gracilis*, *P. angustus*, and *Propolydesmus testaceus* (C. L. Koch, 1847) (Blower 1985, Kime 1990, Pedroli-Christen 1993, Tadler and Thaler 1993).

One isopod (*Trichoniscus alemannicus* Verhoeff, 1917) and one millipede (*C. verhoeffi)* species are known to be restricted to the Alps (Attems 1949, Pedroli-Christen 1993, Schmalfuss, 2003). These cases show that urban areas may support the survival also of native species with a more restricted European distribution.

The isopod *Platyarthrus hoffmannseggii* (Budde-Lund, 1893), a depigmented and blind myrmecophilous species, ubiquitous in ant nests, was frequently sampled in Lucerne. The distribution pattern of such isopods may follow the distribution of their ant hosts.

The intra-European alien (Cochard et al. 2011) *A. nasatum* is commonly introduced to greenhouses across Europe (Schmalfuss 2003). Reports from various cities show that the species can survive in outdoor habitats, too (e.g. Amsterdam: Berg et al. 2008; Budapest: Vilisics and Hornung 2010). The other non-native species was the millipede *O. gracilis,* a true alien of tropical eastern Asian origin. It has been introduced by human activities into European greenhouses (Blower 1985) and seldom survives outdoors in Europe (Pedroli-Christen 1993).

Species compositions, abundances and incidence

Our survey in three Swiss cities resulted in a relatively high species richness and abundance of isopods, while millipedes were captured in lower number of species and individuals.

The overall number of millipede species revealed in the three cities is relatively low (8.7%) compared with the known millipede fauna of Switzerland (127 species; Pedroli-Christen 1993). In temperate Europe the average urban species number is between 14 and 26 (Enghoff 1973, Tischler 1980, Korsós 1992, Korsós et al. 2002, Stoev 2004, Bogyó and Korsós 2009, Riedel et al. 2009), despite the fact that only 9 species were recorded in London by Smith et al. (2006).

Reports on urban isopod and millipede fauna show relatively high species richness in temperate cities as compared to known native, local faunas. Such reports are, however, hardly comparable due to differences in sampled habitats, sampling effort and methodology. Pitfall trapping in parks of the city Debrecen (Hungary) resulted to a 19% (14 species) of the known millipede fauna of Hungary (Bogyó and Korsós 2009). A similar method captured ca. 14% (11 species) of the known millipede fauna of the

Czech Republic from parks of the city Olomouc (Riedel et al. 2009). Pitfall trapping from parks and nearby rural forested areas in Debrecen and Sorø (Denmark) resulted in Isopoda species (Hornung et al. 2007, Vilisics et al. 2007) comprising 11% of Hungarian and ca. 27% of known Danish Isopoda fauna (Meinertz 1964).

Pooled abundances of isopods and millipedes were well over 1000 individuals in each of the above mentioned studies (90 to 120 operating traps for 6 to 9 months). As the number of millipedes in our study was less than 100 individuals, the question arises why abundances were so low in the three Swiss cities? In his review David (2009) has pinpointed the negative effects of habitat loss and low food quality on millipede assemblages, and demography. Furthermore, management practices altering microhabitats (like coarse woody debris and both litter quantity and quality) has great effect on soil macro-invertebrates, including isopods and millipedes. (e.g. Topp et al. 2006, Kappes et al. 2009). The low activity density values in our studied cities may thus be a result of the intensive management such as mowing and removal of plant litter.

The only alien isopod captured, *A. nasatum*, also known as the "greenhouse pillbug", was among the dominating species in Zurich, but it only occurred in 5.7% of the sampling sites (out of the total 36), turning to be one of the rarest species in the city. Similarly, the alien millipede *O. gracilis* (Stoev et al. 2010) showed low incidences, as it appeared in only 3% of the sites in Lucerne, and 12% of Lugano. We assume that these species were either introduced recently in these places or survive in sites which provide special environmental conditions (such as higher annual average temperature), so they may aggregate in high numbers at certain spots of a city, while never establish in others.

The three millipedes (*O. pilosus, B. superus*, *O. gracilis)* occurring in two or more cities are widespread in Europe and occupy rural as well as urban settlements (Blower 1985, Pedroli-Christen 1993, Enghoff 2010). *Ophyiulius pilosus* in Lugano showed the highest incidence of occurrences among the millipedes. We found a somewhat similar trend in isopods, as widely distributed species showed the greatest incidences. Moreover, this result is consistent with Kime (1990) and Voigtländer (2011), who suggested that this species might profit from human activities and disturbances.

Pitfall trapping, as the sampling method employed in this study, is a passive sampling method that has been developed to catch specimens active on the soil and litter surface. It has proven to efficiently represent species richness and activity density data of several arthropod groups (e.g. Araneae, Coleoptera) (Standen 2000). As only a fraction of isopod and millipede species (e.g. isopods: family Trichoniscidae, millipedes: *Geoglomeris* sp., *Polyxenus* sp.) actively move in soil/litter and under bark, pitfall trapping is expected to miss ssome less mobile isopod species that are underrepresented in the present samples. Tuf (2003) has captured the isopod *T. pusillus* with pitfall traps in good numbers, while Vilisics et al. (2007) reported only a few captured specimens in the midst of a dense *T. pusillus* population with the same method. We therefore suppose the pitfall trapping can be useful to assess species diversity and occurrence, while caution remains for abundance data of some species. The data presented here are comparable with other studies that collected isopods and millipedes with the same

method. Moreover, because our study sites were sampled during the same periods and with the same method, the data are comparable among sites and yield a fair description of the relative abundances of the different species during these periods of the season in the study area. In case of future faunistic studies, pitfall trapping should be completed with timed hand search (Vilisics et al. 2011). Such a procedure is expected to provide more complete faunistic results, as well as comparable abundance data for soil and litter dwelling organisms such as isopods and millipedes.

Geographic effects

As with many other invertebrate taxa (e.g. Lepidoptera and Mollusca in IUCN 2011 European Red List), geographic patterns of European isopods and millipedes show a decrease in species richness from southern biodiversity hot-spots to the north. The available literature on millipede and isopod faunas suggests species diversity to decrease roughly by half from Southern Europe to Central Europe, and it further halves towards Fennoscandia (e.g. isopods: Italy: Stoch 2004, Hungary: Hornung et al 2009, Scandinavia: Meinertz 1964); Millipedes: Italy: Stoch 2004, Czech Republic: Tajovský 2001, Scandinavia: Andersson et al. 2005). The Alps are also rich in isopod and millipede species, with cc. 35 endemic isopods (Schmalfuss 2003). Endemic millipedes of Switzerland are known almost exclusively from alpine or subalpine ecosystems (Pedroli-Christen 1993).

Similarities between species compositions hint at the impact of geographical location and distance between cities: the two northern cities (Zurich and Lucerne), which share more species than any other combination of two cities, are only 60 km apart. Lugano is located 170 km south of Lucerne and 210 km south of Zurich, from which it is additionally separated by the Alps. Lugano is already under the influence of Mediterranean climate, which affects the regional flora and fauna.

The common temperate European isopod species (*T. rathkii*, *P. scaber*, *O. asellus*, *P. muscorum*) are among the most common synanthropic species (i.e. species live near humans and benefit from their association with humans and anthropogenous habitats). Common Mediterranean isopod species are also common in urban sites. Besides *A. nasatum*, several other species, such as *Agabiformius lentus* (Budde-Lund, 1885) and *Chaetophiloscia cellaria* (Dollfus, 1884), occur further to the north from their natural range and can survive outdoors (e.g. Cochard et al. 2010). Some xerophilic millipedes (e.g. *B. superus*) show synanthropic trends in their northern range of distribution as well (Voigtländer 2011). One possible reason for this finding is the so-called "heat island effect" (Andreev 2004) which describes a higher average annual temperature in the city core in comparison with the surrounding areas. Introduced isopod and millipede species often find shelter in private and botanical gardens, but their occurrence is typically aggregated to a restricted area within the city (Vilisics and Hornung 2009, Stoev et al. 2010).

Biotic homogenization

Biotic homogenization in cities has been described earlier (e.g. McKinney 2006) as an ongoing process characterized by extinction of local faunal elements and dominance of tolerant species, resulting in incresing similarities in species composition among cities. Szlávecz et al. (2008; unpublished) in their presentation at URBIO2008 (Urban Biodiversity & Design conference in Erfurt, Germany), pinpointed a biotic homogenization process on soil dwelling macro-invertebrates (Annelida, Diplopoda, Isopoda) in major European cities. Based on European literature data from the past 50 years (from e.g. Czech Republic, Hungary, Poland, Romania) the list includes 32 species regarded as „homogenizing" in Europe, including 14 isopods and 18 millipede species. Most of the species are widespread throughout temperate Europe, and several of them were introduced to many parts of the world (Blower 1985, Schmalfuss 2003, Enghoff 2010).

In our study we recorded 16 species mentioned in this list, i.e. 11 isopod species (64.7% of the 17 species found in this study) and 5 millipede species (62.5% of 8). The proportion of widespread isopod and millipede species contributing to homogenization was highest in the largest city, Zurich (81.5% of all 25 species), and lowest in the smaller cities, Lugano (70%) and Lucerne (69%).

Conclusion

Our study showed that urban millipede and isopod assemblages in Switzerland mainly consist of species with wide distribution in Europe. We also showed that cities offer suitable habitats for native and non-native species, with both wide and narrow ecological requirements. Cities under temperate climate showed remarkable differences in their species compositions from the one under Mediterranean influence. This suggests that biogeography plays an important role in shaping isopod and millipede assemblages in the cities.

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RESEARCH ARTICLE

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The diversity of terrestrial isopods in the natural reserve "Saline di Trapani e Paceco" (Crustacea, Isopoda, Oniscidea) in northwestern Sicily

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Abstract

Ecosystems comprising coastal lakes and ponds are important areas for preserving biodiversity. The natural reserve "Saline di Trapani e Paceco" is an interesting natural area in Sicily, formed by the remaining strips of land among salt pans near the coastline. From January 2008 to January 2010, pitfall trapping was conducted in five sampling sites inside the study area. The community of terrestrial isopods was assessed using the main diversity indices. Twenty-four species were collected, only one of them endemic to western Sicily: *Porcellio siculoccidentalis* Viglianisi, Lombardo & Caruso, 1992. Two species are new to Sicily: *Armadilloniscus candidus* Budde-Lund, 1885 and *Armadilloniscus ellipticus* (Harger, 1878). This is high species richness for a single reserve in Sicily. The extended sampling period also allowed us to study species phenology. Most of the species exhibited higher activity in spring than in autumn while some species also exhibited lower activity in the summer. The species richness revealed that the study area is in an acceptable conservation status; Shannon and Pielou indices also confirmed a more or less even distribution of individuals belonging to different species.

Keywords

Isopoda, Oniscidea, Sicily, biodiversity, frequency, phenology

Introduction

Protected areas have restrictions on human activities aimed at preserving biotic and abiotic components of the landscape. Among the natural protected areas, coastal wetlands are particularly important ecosystems for preserving biodiversity (Adam 1990, Allen and Pye 1992). The natural reserve "Saline di Trapani e Paceco" is one of the most important coastal wetlands in Sicily and an acknowledged Site of Community Importance (SIC), Special Protection Area (ZPS), and "Important Bird Area"; it is among the protected wetlands according to Ramsar Convention. The remaining strips of land are particularly interesting and often very small, acting as banks to the salt pans. Many animal and plant species are endemic to this area (Massa et al. 2006, Grammatico and Fici 2008). Much research has been carried out on bird fauna, vegetation, and fauna in salt pans, but little research has investigated the fauna of the remaining strips of land in between the salt pans (Troia 2008).

The aim of this research was to study the diversity of Oniscidea in the natural reserve "Saline di Trapani e Paceco". The specific aims were to study the phenology and frequency of the collected species, and to compare species composition and abundance in sites with similar anthropogenic disturbances to determine if there are differences among them. Oniscidean isopods play an important role in terrestrial ecosystems (Sutton 1980). They are found also in such salty environments that are subject to human pressure due to the traditional activity of the salt pans. The salt in sea water is drawn in solid form from the salt pans and extracted for commercial use. One study has been published on Oniscidea community structure inside protected wetland areas in Sicily (Messina et al. 2011). The present study contributes to our knowledge of this area because "the diversity and abundance of terrestrial arthropods can provide a rich base of information to aid efforts in the conservation of biodiversity and the planning and management of nature reserves" (Kremen et al. 1993, Massa and Ingegnoli 1999). In addition, the isopods at the remaining strips of land in front of the shoreline, if these are well preserved, may play an important role in the local food webs. In fact, the various decomposer arthropods that live in these habitats attract the higher-level vertebrate and invertebrate consumers (Chelazzi et al. 1990).

Material and methods

Study area

The natural reserve "Saline di Trapani e Paceco" (SIC, ITA01007) is located in western Sicily, just south of the town of Trapani (Fig. 1). The reserve has a surface area of 960 ha, consists of a plain characterized by sandy coast with moderate height differences (no more than 5 m above sea level), and is characterized by a large wetland area (80% of the SIC area). The remaining area is divided among areas with intensive human activities (10%), wooded and bushy areas (5%), and agricultural areas (5%). The wetlands are represented by the following categories: groves of reeds, ponds (30 ha), and salt pans (750 ha).

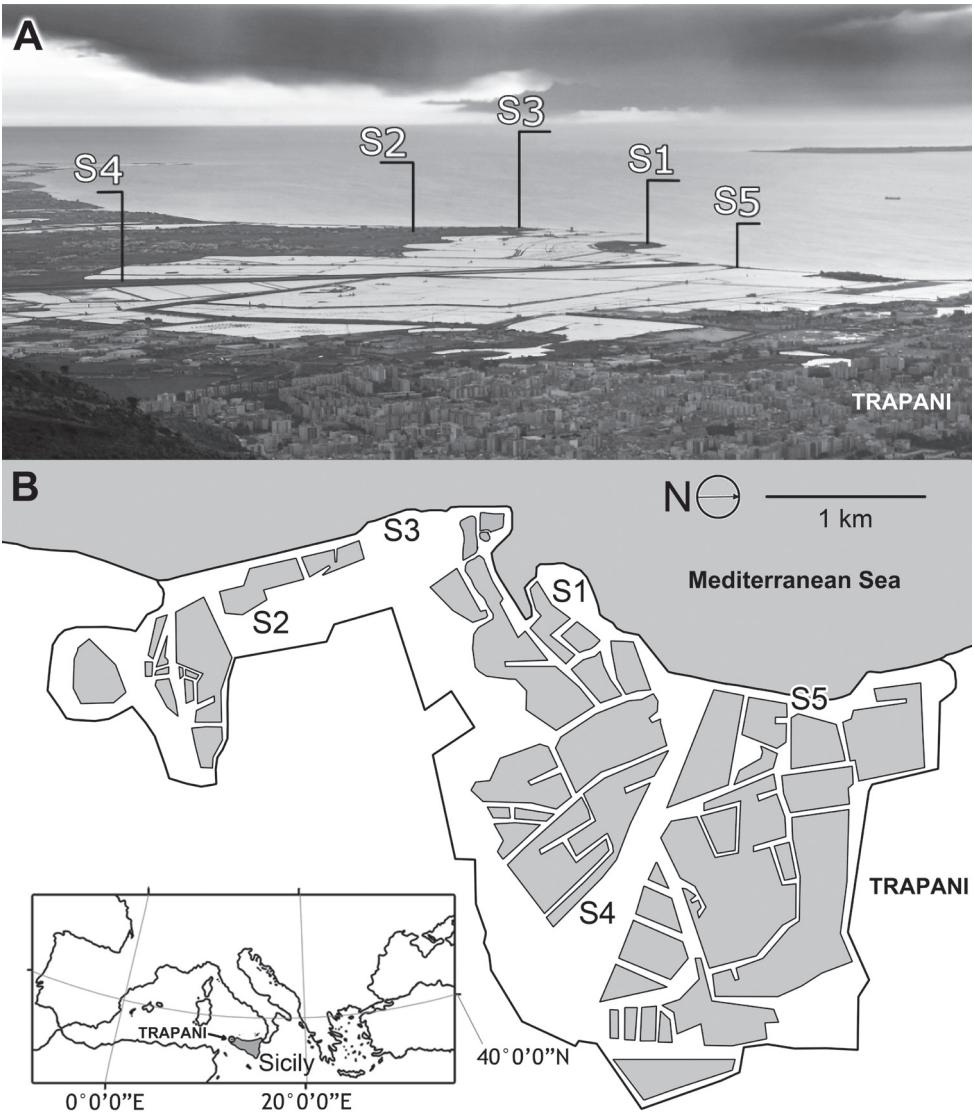

Figure 1. Map of the study area. Sampling sites are indicated. Salt pans are represented by polygons.

Sampling

Pitfall trapping was used as the sampling method. This methodology has provided effective sampling also for isopods (Becker 1975, Fleugge and Levens 1977, Al Dabbagh and Block 1981, Caruso and Zetto Brandmayr 1983). The traps were filled with a saturated water/sodium chloride solution. This solution was used to avoid the attractive effects of formalin and vinegar. The salt functions as a preservative. One advantage of pitfall traps is suitable representation of the qualitative and quantitative

data for the soil fauna (Sutherland 1996). Pitfall trapping does not favor the capture of all isopods present at a site; therefore, species with low mobility are underestimated. However, this sampling method is the best for invertebrate fauna in the soil (New 1999, Brandmayr et al. 2005). The use of pitfall traps also eliminates the problem of different operator abilities when sampling is done by hand. Sampling was conducted from January 2008 to January 2010 at five sampling sites inside the study area, with a transect of pitfall traps traced in each site. Initially, seven traps were placed in each sampling site. In the data analysis we did not consider the traps always devoid of animals because of disturbances (e.g., grazing, hikers, extreme climatic conditions). The same traps were always empty. Thus, the number of considered traps varied at each sampling site. The distance among the traps was almost 20 meters at each sampling site.

Sampling site S1 (WGS84: 37°59'22.4"N, 012°30'01.0"E) consisted of a small island connected to the land by the embankment of the Morana salt pan. Seven traps were recovered in S1 along a transect orthogonal to the coastline. Sampling site S2 (WGS84: 37°58'13.5"N, 12°30'01.4"E) consisted of strips of land comprising the embankments of the Anselmo salt pan, which consists of an uncultivated area. Six traps were recovered in S2 along a transect parallel to the salt pans. Sampling site S3 (WGS84: 37°58'39.6''N, 12°29'44.7''E) was located along the coast in an uncultivated area along the sea. Five traps were recovered in S3 along a transect parallel to the coastline. Sampling site S4 (WGS84: 37°59'19.6"N, 12°31'27.8"E) consisted of a large uncultivated area bordered by a pond on one side and the Baiata canal on the other side. Six traps were recovered in S4 along a transect orthogonal to a pond that borders the area. Sampling site S5 (WGS84: 38°59'53.7"N, 12°30'29.8"E) consisted of a narrow strip of land that separates the salt pans from the sea, a small island connected to the land by an artificial isthmus, which was made to create the embankments of the salt pans. Five traps were recovered in site S5 along a transect traced between the coastline and salt pans. All of the sites are level and characterized by homogeneous vegetation. Visitors to the reserve, salt pan activity, and grazing are disturbance factors for the sampling sites.

The traps were emptied monthly and the material preserved in 70% ethanol. Sampled individuals were identified in the laboratory and the numbers of males, females, and juveniles were counted.

Climate data

The study area is characterized by a temperate Mediterranean climate; rain is concentrated during autumn and winter periods, whereas the climate is hot and dry in summer. Data from 1965-1994 indicated an average annual rainfall of 483 mm. The rainiest season is winter (190.1 mm), followed by autumn (176 mm); the rainiest month is December (75.1 mm). The average annual temperature is 18°C, with a maximum temperature of 41.8°C and minimum of 0.1°C.

Data analysis

The ecological indices used to assess the diversity in each sampling site were: Margalef index ($M = S - 1 / \ln N$, where S is the number of species and N is the total number of individuals), Berger-Parker dominance index ($B = N_{max} / N$, where N_{max} is the number of individuals of the most abundant species), Shannon- Wiener diversity index (H'), and the Pielou evenness index (J') (Magurran 1988). Similarities among sites were calculated using Jaccard's index for presence-absence data and Sörensen's index for quantitative data. The temporal frequency (F), meaning the ratio between the number of times (months) that the presence of a particular species was observed during the 24 months of sampling, was calculated to describe and summarize data on the presence of certain species during the study period. Thus, this index ranged from 0 (the species has never been observed in a site, but has been encountered in other sites) to 24 (the species was sampled at least once a month for every month of the study). Finally, the temporal frequency was calculated by considering all studied sites, both overlapping and individual. Furthermore, as reported by Fallaci et al. (1994), the species were classified as constant ($F \geq 50\%$), accessory ($25 \leq F < 50\%$), accidental ($10 \leq F < 25\%$), or sporadic ($F < 10\%$). For each species with more than 20 individuals (N), the phenological trend was studied by considering the capture frequency, expressed as a percentage, for each sampling month.

Results

Species richness

A total of 24,109 isopod specimens were trapped, representing 24 species and 8 families (Table 1). Species of the family Armadillidiidae were the most common (15956, 66.18%), but Philosciidae (3488, 14.47%), Porcellionidae (2482, 10.29%), Armadillidae (1291, 5.35%), and Halophilosciidae (878, 3.64%) were also well represented. The less represented families were Detonidae (6, 0.02%), Tylidae (5, 0.02%), and Ligiidae (3, 0.01%) (Fig. 2). Among these species, only one is endemic, *Porcellio siculoccidentalis* Viglianisi, Lombardo & Caruso, 1992, which can be considered a neoendemism, found only in western Sicily. Except for *Armadilloniscus candidus* Budde-Lund, 1885 and *A. ellipticus* (Harger, 1878), which were new to the fauna of Sicily, all species collected were already known from the area.

The 24 species found in the present study belong to the following chorological categories. Cosmopolitan: *Porcellionides pruinosus* (Brandt, 1833) and *Porcellio laevis* Latreille, 1804; Mediterranean-Atlantic: *Tylos ponticus* Grebnicki, 1874, *Ligia italica* Fabricius, 1798, *A. ellipticus*, *Halophiloscia couchii* (Kinahan, 1858), *Armadillidium album* Dollfus, 1887, *A. granulatum* Brandt, 1833, and *Armadillo officinalis* Dumeril, 1816; Holomediterranean: *Chaetophiloscia elongata* (Dollfus, 1884), *Agabiformius len-*

Table 1. Species and number of catches for each sampling site; diversity indices values for each sampling site are also reported.

Species	S1	S2	S3	S4	S5	S1-S5
Family Tylidae						
Tylos ponticus Grebnicki, 1874	2				3	5
Family Ligiidae						
Ligia italica Fabricius, 1798	3					3
Family Detonidae						
Armadilloniscus candidus Budde-Lund, 1885	1		1		1	3
Armadilloniscus ellipticus (Harger, 1878)			2		1	3
Family Halophilosciidae						
Halophiloscia couchii (Kinahan, 1858)	330	1	520		2	853
Halophiloscia hirsuta Verhoeff, 1928			20		1	21
Stenophiloscia glarearum Verhoeff, 1908	1		1		2	4
Family Philosciidae						
Chaetophiloscia elongata (Dollfus, 1884)	218	368	1664	1043	195	3488
Family Porcellionidae						
Porcellionides pruinosus (Brandt, 1833)				8		8
Porcellionides sexfasciatus (Budde-Lund, 1885)		1				1
Acaeroplastes melanurus (Budde-Lund, 1885)	28	6	16	12		62
Agabiformius lentus (Budde-Lund, 1885)	11	13	21	18	10	73
Agabiformius obtusus (Budde-Lund, 1909)				4		4
Leptotrichus panzerii (Audouin, 1826)	85	727	357	4	267	1440
Lucasius pallidus (Budde-Lund, 1885)	1		1	2		4
Mica tardus (Budde-Lund, 1885)	4			4		8
Porcellio albicornis (Dollfus,1896)	4	6	10	8	5	33
Porcellio laevis Latreille, 1804	66	47	486	178	27	804
Porcellio siculoccidentalis Viglianisi, Lombardo, Caruso, 1992	45					45
Family Armadillidiidae						
Armadillidium album Dollfus, 1887					5	5
Armadillidium badium Budde-Lund, 1885	887	23	174	510	2	1596
Armadillidium decorum Brandt, 1833	1			424	1	426
Armadillidium granulatum Brandt, 1833	14	540	1525	3	11847	13929
Family Armadillidae						
Armadillo officinalis Dumeril, 1816		7	380	895	9	1291
Total catch (number of individuals)	**1701**	**1739**	**5178**	**3113**	**12378**	**24109**
Species richness	17	11	15	14	16	24
Margalef index (M)	2.1510	1.3400	1.6370	1.6160	1.5920	2.2790
Shannon-Wiener index (H')	1.4980	1.3180	1.7500	1.5750	0.2301	1.4810
Pielou's evenness index (J')	0.5286	0.5495	0.6460	0.5969	0.0830	0.4661
Berger-Parker index	0.5215	0.4181	0.3214	0.3350	0.9571	0.5778

tus (Budde-Lund, 1885), *A. obtusus* (Budde-Lund, 1909), and *Leptotrichus panzerii* (Audouin, 1826); North-Mediterranean: *Stenophiloscia glarearum* Verhoeff, 1908 and *Halophiloscia hirsuta* Verhoeff, 1928; West-Mediterranean-Atlantic: *Armadilloniscus candidus*, *Porcellionides sexfasciatus* (Budde-Lund, 1885), *Lucasius pallidus* (Budde-Lund, 1885), and *Acaeroplastes melanurus* (Budde-Lund, 1885); South-Mediterrane-

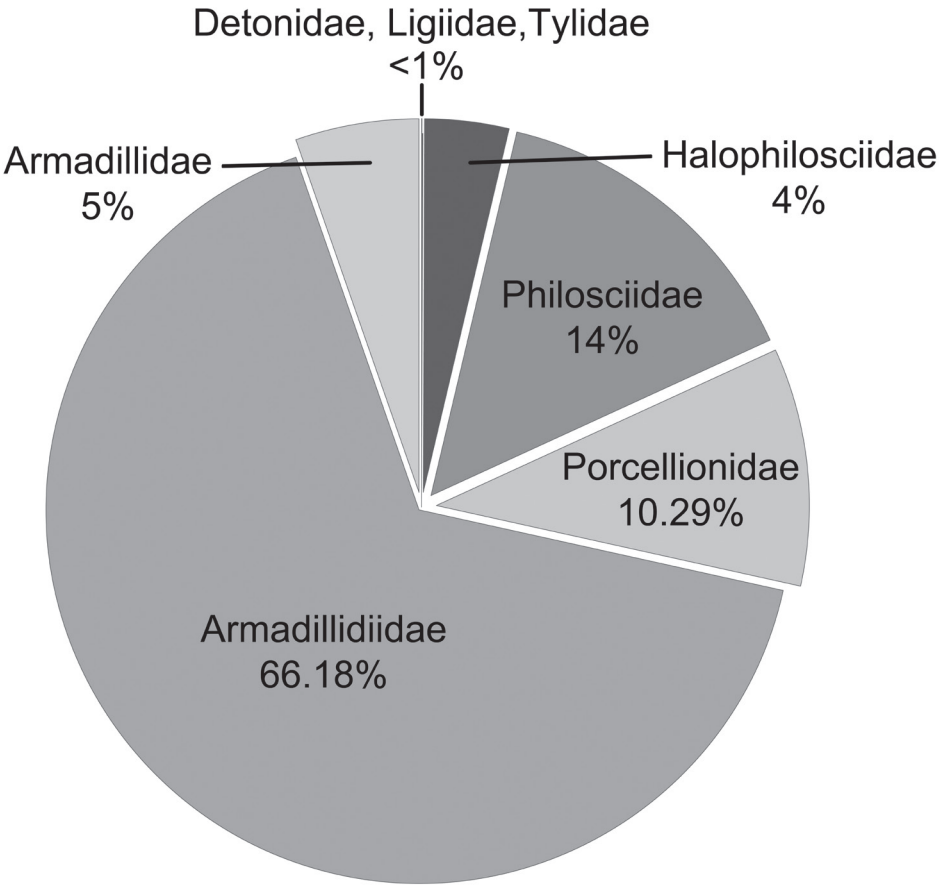

Figure 2. Total frequency (%) of catches for the observed families.

an: *Mica tardus* (Budde-Lund, 1885) and *Porcellio albicornis* (Dollfus,1896); Calabrian-Sicilian-South-Mediterranean: *Armadillidium badium* Budde-Lund, 1885 and *A. decorum* Brandt, 1833; and endemic species: *Porcellio siculoccidentalis*. (Caruso 1973, Schmalfuss 2003, Taiti and Ferrara 1996).

Species assemblages

The total number of individuals sampled in each site ranged from a minimum of 1,701 in S1 to a maximum of 12,378 in S5 (Table 1). Species richness at each site ranged from 11 in S2 (M = 1.34) to 17 in S1 (M = 2.15). *Chaetophiloscia elongata* was the dominant species in two sites (S3 and S4) (Table 1). In S3, *C. elongata* and *Armadillidium granulatum* were co-dominant, whereas a high degree of dominance was restricted to single species in the other sites. *Leptotrichus panzerii* was dominant in S2, *Armadillidium badium* was dominant in S1, and *A. granulatum* was dominant in S5 (Table 1).

Shannon and Pielou indices had the lowest values in S5 and highest values in S3 (Table 1). Sampling site S3 had few dominant species (H' = 1.75; J' = 0.65) and was represented by 15 species. In S5 (H' = 0.23; J' = 0.08), even though there was greater species richness, high dominance was found due to the massive presence of *A. granulatum.*

The Jaccard index ranged from 0.43 to 0.72, with sites S3 and S5 being the most similar. The Sörensen index ranged from 0.03 to 0.52. The most similar pair of sites was S1 and S4, and the less similar was S4-S5 (Table 2).

Table 2. Similarity analysis based on the Jaccard index (below the diagonal) and Sörensen index (above the diagonal).

	S1	S2	S3	S4	S5
		0.24	0.27	0.52	0.05
S2	0.47		0.40	0.20	0.15
S3	0.60	0.63		0.44	0.23
S4	0.55	0.56	0.53		0.03
S5	0.57	0.50	0.72	0.43	

Temporal frequency and phenology

Analysis of the temporal frequency in the overall study area showed that 11 species were constant (F ≥ 50%), only one species was accessory (25 ≤ F < 50%), 6 were accidental (10 ≤ F < 25%), and 6 were sporadic (F < 10%). The frequency category for each species did not change in the different sites in regards to the accidental and sporadic species, whereas constant species were not the same among sites (Table 3).

Table 3. Temporal frequency (%) analysis for each sampling site.

S1		
Constant	*Armadillidium badium*	95.65
	Leptotrichus panzerii	86.96
	Chaetophiloscia elongata	69.57
	Porcellio laevis	65.22
	Porcellio siculoccidentalis	65.22
	Halophiloscia couchii	52.17
Accessory	*Acaeroplastes melanurus*	39.13
	Agabiformius lentus	26.09
	Armadillidium granulatum	26.09
Accidental	*Porcellio albicornis*	17.39
Sporadic	*Tylos ponticus*	8.70
	Mica tardus	8.70
	Ligia italica	4.35
	Armadilloniscus candidus	4.35
	Stenophiloscia glarearum	4.35
	Lucasius pallidus	4.35
	Armadillidium decorum	4.35

S2		
Constant	*Leptotrichus panzerii*	91.67
	Armadillidium granulatum	79.17
	Chaetophiloscia elongata	70.83
	Porcellio laevis	54.17
Accessory	*Agabiformius lentus*	41.67
	Armadillidium badium	37.50
Accidental	*Acaeroplastes melanurus*	20.83
	Armadillo officinalis	20.83
	Porcellio albicornis	16.67
Sporadic	*Halophiloscia couchii*	4.17
	Porcellionides sexfasciatus	4.17
S3		
Constant	*Chaetophiloscia elongata*	95.83
	Porcellio laevis	95.83
	Armadillidium granulatum	95.83
	Leptotrichus panzerii	83.33
	Armadillo officinalis	70.83

	Halophiloscia couchii	66.67
	Armadillidium badium	58.33
	Agabiformius lentus	54.17
Accessory	*Acaeroplastes melanurus*	45.83
Accidental	*Porcellio albicornis*	12.50
Sporadic	*Halophiloscia hirsuta*	8.33
	Armadilloniscus candidus	4.17
	Armadilloniscus ellipticus	4.17
	Stenophiloscia glarearum	4.17
	Lucasius pallidus	4.17
S4		
Constant	*Chaetophiloscia elongata*	95.83
	Armadillidium badium	95.83
	Armadillidium decorum	87.50
	Porcellio laevis	83.33
	Armadillo officinalis	83.33
Accessory	*Acaeroplastes melanurus*	37.50
Accidental	*Agabiformius lentus*	20.83
Sporadic	*Leptotrichus panzerii*	16.67
	Porcellionides pruinosus	12.50
	Porcellio albicornis	12.50
	Armadillidium granulatum	12.50
	Agabiformius obtusus	4.17
	Lucasius pallidus	4.17
	Mica tardus	4.17
S5		
Constant	*Armadillidium granulatum*	100.00
	Leptotrichus panzerii	78.26
	Porcellio laevis	56.52
Accessory	*Chaetophiloscia elongata*	47.83
	Armadillo officinalis	34.78
	Agabiformius lentus	26.09
Accidental	*Armadillidium album*	17.39
Sporadic	*Tylos ponticus*	8.70
	Halophiloscia couchii	8.70
	Porcellio albicornis	8.70
	Armadillidium badium	8.70
	Armadilloniscus candidus	4.35
	Armadilloniscus ellipticus	4.35
	Halophiloscia hirsuta	4.35
	Stenophiloscia glarearum	4.35
	Armadillidium decorum	4.35
S1 – S5		
Constant	*Chaetophiloscia elongata*	100.00
	Porcellio laevis	100.00
	Armadillidium granulatum	100.00
	Leptotrichus panzerii	95.83
	Armadillidium badium	95.83
	Armadillidium decorum	87.50
	Armadillo officinalis	87.50
	Agabiformius lentus	79.17
	Halophiloscia couchii	75.00
	Acaeroplastes melanurus	75.00
	Porcellio siculoccidentalis	62.50
Accessory	*Porcellio albicornis*	41.67
Accidental	*Armadillidium album*	16.67
S1 – S5 continued		
Sporadic	*Tylos ponticus*	12.50
	Halophiloscia hirsuta	12.50
	Stenophiloscia glarearum	12.50
	Porcellionides pruinosus	12.50
	Mica tardus	12.50
	Armadilloniscus ellipticus	8.33
	Lucasius pallidus	8.33
	Ligia italica	4.17
	Armadilloniscus candidus	4.17
	Porcellionides sexfasciatus	4.17
	Agabiformius obtusus	4.17

Among the constant species only three exhibited a frequency of 100%: *C. elongata*, *P. laevis* Latreille, 1804, and *A. granulatum*. *P. laevis* was constant at all sites (Table 3); *C. elongata*, *A. granulatum*, *L. panzerii*, *A. badium*, *A. officinalis*, *A. lentus* and *H. couchii* (Kinahan, 1858) were constant species in S3, though they sometimes changed frequency category in the other sites; *C. elongata* was accessory in S5, *A. granulatum* was accessory in S1 and accidental in S4, *L. panzerii* was accidental in S4, *A. badium* was accidental in S2 and sporadic in S5, *A. officinalis* was accidental in S2 and accessory in S5, *A. lentus* was accidental in S1 and S4 and accessory in S2 and S5, and *H. couchii* was sporadic in S2 and S5. Two species, *P. siculoccidentalis* and *A. decorum* were constant in only one sampling site, S1 and S4, respectively. *Acaeroplastes melanurus* was not constant in any site. The number of sporadic species varied from two in S2 to nine in S5 (Table 3).

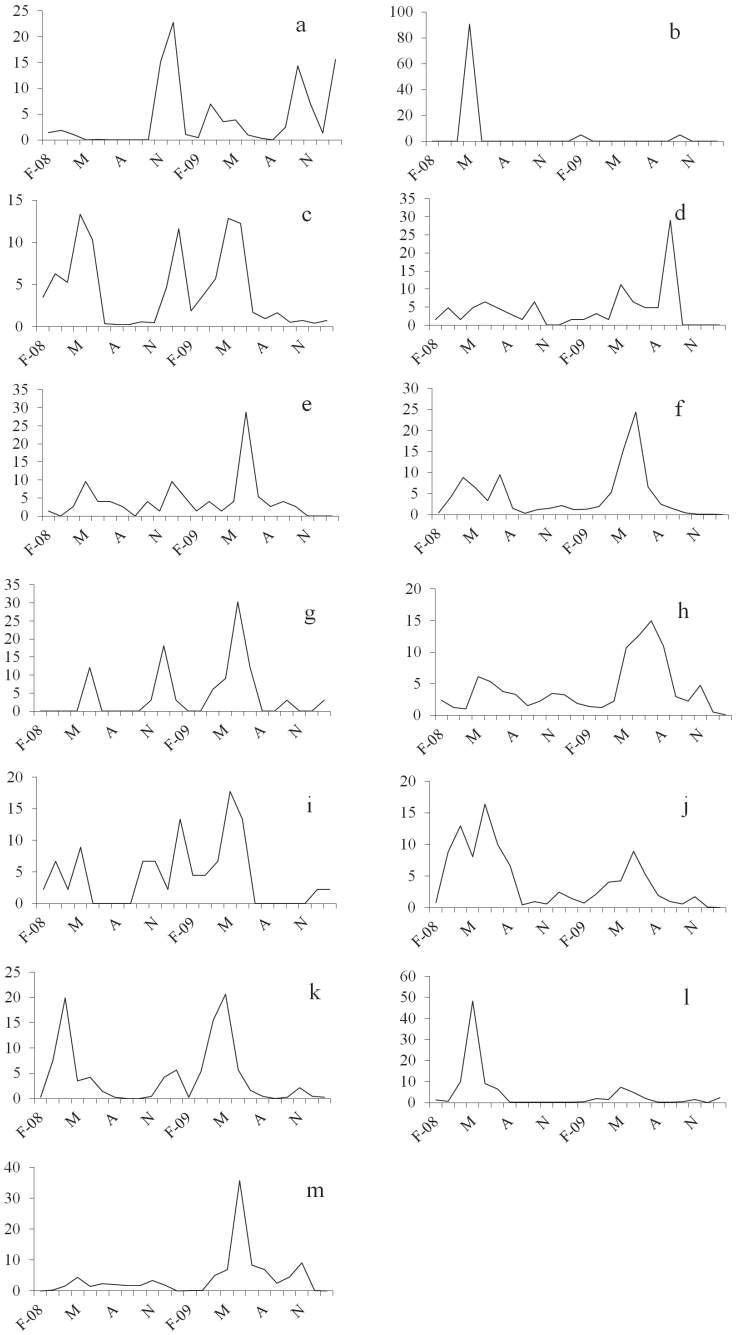

Figure 3. Frequency of catches (%) for each sampling month. **a** *Halophiloscia couchii* (N= 853) **b** *Halophiloscia hirsuta* (N= 21) c *Chaetophiloscia elongata* (N= 3488) **d** *Acaeroplastes melanurus* (N= 62) **e** *Agabiformius lentus* (N= 73) f *Leptotrichus panzerii* (N= 1440) **g** *Porcellio albicornis* (N= 33) **h** *Porcellio laevis* (N= 804) **i** *Porcellio siculoccidentalis* (N= 45) **j** *Armadillidium badium* (N= 1596) **k** *Armadillidium decorum* (N= 426) **l** *Armadillidium granulatum* (N= 13929) **m** *Armadillo officinalis* (N= 1291).

On the basis of the collected species and their 'abundance', we were able to evaluate the distribution of species (N >20) in the five sampling sites and to study the activity trend during the sampling period. *Halophiloscia couchii* was represented by a fairly large number of individuals (N=853), which were present more in S1 (38.7%) and S3 (61%) (Table 1). We observed a period of weak activity in the spring with a minimum during the driest periods. During the autumn, the activity intensified with a peak in December that was anticipated in October during the second year (Fig. 3a). *Chaetophiloscia elongata* was represented by a large number of individuals (N=3488), which were found mostly in S3 (48%). The species showed an activity period from February to June in both years. After the summer in which there was no activity, the curve showed a rise with a peak in late autumn (Fig. 3c). *Agabiformius lentus* was represented by a small number of individuals (N=73), which were distributed equally in the five sampling sites. Generally, this species exhibited weak activity that intensified slightly in May and December of the first year and June of the second year (Fig. 3e). *Porcellio albicornis* was represented by only 33 specimens equally distributed in the five sampling sites. The phenological curve showed a peak of activity in late spring, no activity in the summer, and a second smaller peak in autumn (Fig. 3g). *Porcellio siculoccidentalis* was represented by a small number of individuals (N=45) and found only in S1 (Fig. 3i). This species exhibited continuous activity in all months of the year except the driest periods, when it was completely inactive. *Armadillidium badium* was represented by 1596 individuals, many of which were present in S1 (55.6%). The activity of this species started in March, reached a peak in June, and decreased during the summer months. In winter, a second small peak was observed (Fig. 3j). *Armadillidium decorum* was represented by 426 individuals, among which, 424 individuals were present in S4 (99.5%). The phenological curve showed a bimodal trend in both years, with a peak of activity in April and January of the first year and November of the second year (Fig. 3k). *Armadillidium granulatum* was the most represented species with 13,929 individuals, many of which (N=11,847) were collected in S5 (85%). This species showed early activity in March, a peak in late spring, and reduced activity in August (Fig. 3l). *Acaeroplastes melanurus* and *L. panzerii* exhibited activity in almost all months of the year, except November and December (Figs. 3d-3f). *Acaeroplastes melanurus* was represented by 62 individuals, many of which were present in S1 (45.2%). *Leptotrichus panzerii* was represented by 1440 individuals, which were collected mostly in S2 (50.5%). *Porcellio laevis* and *A. officinalis* exhibited activity throughout the sampling period with two peaks, in the spring and summer (Figs. 3h-4e). *Porcellio laevis* was represented by 804 individuals, which were collected mostly in S3 (60.4%). *Armadillo officinalis* was represented by 1291 individuals, which were collected mostly in S4 (69.3%) (Fig. 3m).

Discussion and conclusion

Species richness

In the study area, we found 27% of the total number of species known in Sicily (90). The 24 species collected were found in very similar habitats, whereas the Sicilian spe-

cies come from all kinds of biotope (e.g., caves, mountains, woods, etc.). Comparing these data with other research concerning the diversity of terrestrial isopods in the coastal wetland of Vendicari (Natural Reserve in southeastern Sicily, Syracuse province) (Messina et al. 2011), the number of species was nearly the same. Indeed 23 species were found in Vendicari. The two areas have only 13 species in common (*H. couchii*, *H*, *hirsuta*, *C. elongata*, *P. pruinosus*, *P. sexfasciatus*, *A. melanurus*, *A. lentus*, *A. obtusus*, *L. panzerii*, *P. laevis*, *A. badium*, *A. granulatum*, and *A. officinalis*). The area of Vendicari seems to be in a better condition than the "Saline di Trapani e Paceco" due to the presence of new species belonging to the genera *Bathytropa*, *Spelaeoniscus*, and *Haplophthalmus* , the former two of which are endemic to the area and belong to genera known to be highly sensitive even to low levels of environmental degradation. Indeed, species belonging to *Bathytropa* and *Spelaeoniscus* live almost exclusively in undisturbed habitats (Caruso and Lombardo 1976).

The species richness in "Saline di Trapani e Paceco" is significantly higher than that of other Mediterranean wetland sites. In coastal wetlands in Tunisia, 14 species were collected (Khemaissia et al. 2011), eight of which (*L. italica*, *C. elongata*, *P. pruinosus*, *P. sexfasciatus*, *L. panzerii*, *P. laevis*, *A. granulatum*, and *A. officinalis)* in common with the present study area. In the Berkoukech area (north-west of Tunisia), 12 species of terrestrial isopods were collected (Achouri et al. 2008), five of which (*C. elongata*, *P. pruinosus*, *P. sexfasciatus*, *L. panzerii*, and *A. album*) are also present in our study area. In the Moula-Bouterfess area, 11 species were collected (Hamaïed-Melki et al. 2010), only two of which (*C. elongata* and *P. sexfasciatus*) in common with our study. Comparisons with these data, however, are spurious because of different sampling methods.

Considering the ecological requirements of the 24 species, they can be grouped as littoral halophilic (*T. ponticus*, *L. italica*, *A. candidus*, *A. ellipticus*, *H. couchii*, *H. hirsuta*, *S. glarearum*, and *A. album*), coastal (*A. melanurus*, *A. obtusus*, *P. sexfasciatus*, and *A. granulatum*), sabulicolous (*A. lentus* and *L. panzerii*), xerophilic (*A. officinalis*); pratinicolous (*M. tardus*, *L. pallidus*, *A. badium*, and *A. decorum*), humicolous (*C. elongata* and *P. siculoccidentalis*), anthropophilic (*P. pruinosus* and *P. laevis*), and myrmecophilous species (*P. albicornis*).

Species assemblages

All sampling sites except S2 have a high and comparable number of species, but vary in composition. In sampling sites S1, S3, and S5 we found halophilic species whereas in S2 and S4 these species were absent, except for *H. couchii*, which was found in S2 at the edge of the salt pans. As indicated by the diversity and evenness indices, a relatively even distribution of individuals among species can be seen in four of the sampling sites. S5 is an exception, due to the very high population of *A. granulatum*, which is always present with many individuals. Other cases of population explosion are known in the literature (Warburg 1993), such as for *Armadillidium vulgare* (Latreille, 1804) in

North America (Hatch 1947) and *A. granulatum* in Panarea, which covered the streets of the island during the night (Caruso 1968). An enormous population explosion of *A. decorum* invaded the streets and houses of the town of Collesano (PA) with millions of individuals in the spring of 1998.

Comparing the similarity values (Jaccard index) among the sampling sites, S3 and S5 were qualitatively more similar, having 13 species in common, including strictly halophilic species *A. candidus*, *A. ellipticus*, *H. couchii*, *H. hirsuta*, and *S. glarearum* and the coastal species *A. granulatum*. Halophilic species determine the qualitative similarity among all sites. The less similar sites are S4 and S5 (9 species in common) because S4 lacks halophilic species and is richer in species that prefer wet and open areas, such as *L. pallidus* and *M. tardus.*

The quantitative Sörensen index showed generally low values. S1 and S4 were fairly similar, whereas S4 and S5 were less similar, as for Jaccard's index.

Temporal frequency and phenology

Analysis of the temporal frequency of the species in each site showed constancy of species tied to specific habitats. For example, *A. badium* which lives in grasslands and prefers open areas (Caruso and Lombardo 1982), was constant in S1, where it was found in a large area with low and sparse vegetation, whereas the species *C. elongata* was constant in S3 and S4, which are both environments with a high level of humidity. A high number of sporadic species was collected in S5; this site is characterized by a narrow strip of land (50 m) between the coastline and salt pans. A majority of the sporadic species are halophilic species typical of the habitats present in this site. Such low frequency values can be explained by the fact that almost all species, including *A. candidus*, *A. ellipticus*, *H. couchii*, *H. hirsuta*, *S. glarearum*, and *A. album*, live near the shoreline and rarely move away. The lack of *T. ponticus* is strange because migration from the sea to inland and vice versa occurs every night, up to 200 meters from the shoreline (Pardi 1955, Tongiorgi 1969, Alicata et al. 1982).

Most of the species, specifically *H. couchii*, *C. elongata*, *A. lentus*, *P. albicornis*, *P. siculoccidentalis*, *A. badium*, *A. decorum*, and *A. granulatum*, exhibit high activity in spring and decreased activity during the driest months. A second peak occurs in autumn, perhaps corresponding to the activity of the spring generation. This general trend varies for *A. melanurus*, *L. panzerii*, *P. laevis*, and *A. officinalis*, which exhibit low activity in the summer.

The different types of sampling methods used in other studies of similar habitats (Achouri et al. 2008, Hamaïed-Melki et al. 2010, Kemaissia et al. 2011, Hamaïed-Melki et al. 2011), does not permit comparison of phenological data. In agreement with Colombini et al. (2002), though, we found that *H. couchii* is more active in April and October. Comparing the results obtained here with those that emerged from similar research carried out in the natural reserve of Vendicari (Messina et al. 2011), we verified that the common species to both areas have an annual activity trend with two

peaks in the spring and autumn. The activity periods of *C. elongata*, *A. badium*, and *A. granulatum* do not coincide; in Vendicari *C. elongata* is most abundant in the summer, whereas *A. badium* and *A. granulatum* peak in autumn.

In the present study area, no species of special conservation concern has been found. Nevertheless, and despite the fact that the area is disturbed by human activity at the salt pans, it can be considered of a good environmental quality and of some conservation interest. This conclusion can be inferred by the relatively high number of isopod species and the generally even distribution among them. The only exceptional case was *A. granulatum* in S5, with a population explosion that could be due among other factors to a drastic decrease in predators due to human activities.

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Feeding rates of *Balloniscus sellowii* (Crustacea, Isopoda, Oniscidea): the effect of leaf litter decomposition and its relation to the phenolic and flavonoid content

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Abstract

The goal of this study was to compare the feeding rates of *Balloniscus sellowii* on leaves of different decomposition stages according to their phenolic and flavonoid content. Leaves from the visually most abundant plants were offered to isopods collected from the same source site. *Schinus terebinthifolius*, the plant species consumed at the highest rate, was used to verify feeding rates at different decomposition stages. Green leaves were left to decompose for one, two, or three months, and then were offered to isopods. The total phenolic and flavonoid contents were determined for all decomposition stages. Consumption and egestion rates increased throughout decomposition, were highest for two-month-old leaves, and decreased again in the third month. The assimilation rate was highest for green leaves. The mode time of passage through the gut was two hours for all treatments. Ingestion of leaves occurred after two or three days for green leaves, and on the same day for one-, two- and three-month-old leaves. The speed of passage of leaves with different decomposition stages through the gut does not differ significantly when animals are fed continuously. However, it is possible that the amount retained in the gut during starvation differs depending on food quality. The digestibility value was corrected using a second food source to empty the gut of previously ingested food, so that all of the food from the experiment was egested. The digest-

ibility value was highest for green leaves, whereas it was approximately 20% for all other stages. This was expected given that digestibility declines during decomposition as the metabolite content of the leaves decreases. The phenolic content was highest in the green leaves and lowest in three-month-old leaves. The flavonoid content was highest in green leaves and lowest after two months of decomposition. Animals ingested more phenolics when consumption was highest. The estimated amount of ingested flavonoids followed the same trend as assimilation rate. Flavonoids accounted for a large portion of total phenolics, and the estimated amount of flavonoids consumed was similar for one-, two- and three-month-old leaves. Our results suggest that the high phenolic and flavonoid concentrations in green leaves are feeding deterrents. Isopods may discriminate among concentrations of flavonoids and modify their consumption rates to maintain their intake of flavonoids when ingesting leaves with lower flavonoid content.

Keywords
Woodlice, digestibility, total phenolics, flavonoid concentration, consumption rate, assimilation rate

Introduction

Litter dynamics are of great importance in ecosystem functioning and are influenced by many different organisms. Isopods, earthworms, lumbricids, diplopods, dipteran larvae, and termites are detritivores of organic soil litter that play a major role in the cycling of nutrients, which is an important ecosystem service. Detritivores have low assimilation efficiency (Szláveczs and Pobozny 1995), and thus contribute to leaf litter decomposition indirectly by returning large amounts of consumed litter as feces (Quadros and Araujo 2008), which provides increased surfaces that are readily colonized by microbial populations (Hassall et al. 1987, Loureiro et al. 2006).

Detritivores exhibit feeding preferences that may be related to leaf senescence (Wieser 1984, Hassall et al. 1987, Yeates and Barmuta 1999), the nutrient content of food (Graça et al. 2001), microbial colonization (Gunnarsson 1987, Kautz et al. 2000, Zimmer et al. 2003, Ihnen and Zimmer 2008), and the presence of unpalatable or indigestible compounds (Cameron and LaPoint 1978, Hassall and Rushton 1984, Target et al. 1986, Sousa et al. 1998, Canhoto and Graça 1999, Lambdon and Hassall 2005). The contribution of isopods to decomposition depends on leaf litter degradation and may be influenced by food preference (Van Wensem et al. 1993).

Frequent switching between different types of food and regulating the intake of specific defensive chemicals are behavioral mechanisms used to accommodate for chemically defended foods (Glendinning 2007). Whenever possible, isopods ingest decayed plant material of different plant types (Wieser 1984). However, experiments of feeding preference between more than two food sources are difficult to analyze (Wieser 1984, Peterson and Renaud 1989). Although the feeding rates of isopods on different plant species have been studied extensively, the reported digestibility efficiency values remain controversial for high and low quality food. Many researchers hypothesize that some foods might present high digestibility values due to a slower passage through the gut, which decreases fecal production (Souza et al. 1998, Loureiro et al. 2006). Changes in the chemical composition of the litter due to decomposition increase its palatability to

detritivores (Cameron and LaPoint 1978, Neuhauser and Hartenstein 1978, Rushton and Hassall 1983, Hassall and Rushton 1984, Hassall et al. 1987, Wan Wensen et al. 1993) because the phenolic content decreases during leaf senescence due to the action of microorganisms and leaching (Zimmer 1999, Zimmer 2002a).

Phenolics are thought to play a fundamental role in the chemical defense of plants against herbivores and pathogens (Harborne 1993, Kefeli et al. 2003, Gould and Lister 2006), although their effects are still debated and not fully understood (Appel 1993, Johnson and Felton 2001). The total phenolic content varies with plant growth and abiotic factors, such as temperature and radiation (Salgado et al. 2008), and may affect microbial decomposers since most phenolics remain present during leaf senescence and after death (Bärlocher and Graça 2005). Although poorly understood, the existence of a phenolic cycle in the plant-soil system has been recorded (Kefeli et al. 2003), and differences in the composition and concentration of resin acids and phenolics during leaf and needle litter senescence are known (Kuiters and Sarink 1986, Kainulainen and Holopainen 2002). For example, Hassall and Rushton (1984) observed a negative correlation between isopod feeding preference and the phenol content. In contrast, Neuhauser and Hartenstein (1978) found no relationship between leaf palatability and total phenolic content, and Kasurinen et al. (2007) found a weak or inconsistent correlation between detritivore feeding performance and chemical parameters of leaf litter.

The ability to digest phenolic polymers such as tannins and lignin is essential in the use of litter (Zimmer 2002b), and studies have demonstrated that isopods are capable of oxidizing (Stevenson 1961, Zimmer and Topp 1998, Zimmer 1999b, Zimmer et al. 2002) or hydrolyzing ingested phenolics (Zimmer 1999, Zimmer et al. 2002). For example, Cameron and LaPoint (1978) observed senescence-related decreased mortality and increased leaf consumption after leaching of tannins in *Armadillidium vulgare* (Latreille, 1804), and suggested that litter resources cannot be used immediately after leaf fall due to the chemical and mechanical defenses of plants.

Most studies examining detritivore feeding and phenolics have explored the relationship with total phenolics (concentrations at which animals avoid feeding), lignin content related to toughness (not included in the total phenolic determination since it is a non-soluble phenolic) (Graça and Zimmer 2005), or the capacity of tannins (polyphenolics) to inhibit enzyme catalyzed reactions or to bind and precipitate proteins (Graça and Bärlocher 2005). Conversely, for insects, flavonoids, which are phenolics commonly found in plants, are related to feeding deterrence and might interfere with feeding, molting, and reproduction (Oberdörster et al. 2001, Simmonds 2001, Boué and Raina 2003, Gould and Lister 2006). Some flavonoids play an important role in the protection of plants from harmful ultraviolet (UV)-B levels (Gould and Lister 2006), and several classes of flavonoids show antioxidant activity towards a variety of readily oxidizable compounds (Gryglewski et al. 1987, Dixon and Steele 1999, Zhishen et al. 1999). However, the effects of ingesting flavonoids by other groups of soil invertebrates, such as terrestrial isopods, have received little attention. Studies of isopod nutrition have mainly been conducted in Europe (Rushton and Hassall 1983, Gunnarsson 1987, Szlávecz and Pobozny 1995, Souza et al. 1998, Zimmer 2002, Ihnen and Zimmer 2008) using species such as *Porcellio* Latreille, 1804 and

Armadillidium Brandt, 1833, which exhibit worldwide distributions. Studies with neotropical species are relatively uncommon and should be encouraged.

The goal of this study was to observe how two interconnected food parameters (phenolic content and decomposition stage) affect feeding rates (consumption, egestion, and assimilation rates, as well as digestibility efficiency) of detritivores using the neotropical terrestrial isopod *Balloniscus sellowii* (Brandt, 1833) as a model. We relate the total phenolic content to feeding rates and examine the flavonoid contents, which constitute a specific group of phenolics that are known to deter insects. Finally, we test a new method of calculating digestibility (assimilation efficiency) that takes into consideration food retention in the gut.

Material and methods

Species and source site

The species *Balloniscus sellowii* (Brandt, 1833) is common in Southern Brazil, Uruguay, and the region surrounding Buenos Aires in Argentina (Schmalfuss 2003). Specimens of *B. sellowii* were collected in a urban area of Porto Alegre, Rio Grande do Sul, southern Brazil and kept in laboratory conditions at 20 ± 1ºC under a 12:12 (light:dark) photoperiod. Only intermolt animals heavier than 25 mg were used in the experiments, excluding ovigerous females.

The source-site consisted of an area where animals were abundant and there were trees characteristic of pioneer vegetation colonization. The three most abundant plant species in the site were *Lithraea brasiliensis* Marchand (Anacardiaceae), *Ricinus communis* Linnaeus (Euphorbiaceae), and *Schinus terebinthifolius* Raddi (Anacardiaceae).

Feeding preference

Leaves from the visually most abundant local plants were offered to isopods to verify feeding preference based on the highest consumption. Green leaves from three different plant species were removed from branches and placed into litter bags (10 x 15 cm) fastened to the soil for decomposition in loco for 14 days. Leaves were then transported to the laboratory and circles of 18 mm in diameter were cut and oven dried at 60ºC for 48 hours. The discs were weighed (Gibertini E425-B) and remoistened with distilled water before being offered to animals for one week in individual units consisting of 8 cm diameter plastic containers with moist plaster of Paris covered with a net to minimize coprophagy. The treatments consisted of 10 units with one leaf disc of each plant species with one isopod, and five animal free control units. Animals were kept without food for two days prior to and after the experiment to empty gut contents. After the experiment, the remaining plant material and feces were oven dried and reweighed, and consumption rates were

calculated. The control group consisted of units containing leaves and no animals, such that the mean percentage of leaf weight lost due to autogenic changes (weight lost independent of the action of consumers) was subtracted from the amount of plant consumed. The plant species that was consumed at the highest rate was used to verify feeding rates on leaves at different stages of decomposition, as well as the phenolic and flavonoid contents of the leaves.

Feeding rates on leaves in different stages of decomposition

Green leaves were collected from branches at the same site and placed into 20 litter bags. The litter bags were collected after one, two, and three months of decomposition. These leaves were then taken to laboratory along with additional green leaves that had been collected from the branches when litter bags were placed in the soil, and offered to animals. Oven dried leaves from each decomposition stage were stored under refrigeration for phenolic and flavonoid content analysis.

For each unit, two or three discs of 18 mm (approximated amount for the third month of decomposition, at which point the leaves were very fragmented) were oven dried, weighed, remoistened with distilled water, and offered to individual animals for 10 days. The remaining leaves and feces were collected from the units, oven dried, and weighed after the experiment to calculate feeding rates. We performed 20 repetitions with one animal per unit and 20 control repetitions for each stage of decomposition.

Consumption rates were calculated as the total mg of ingested leaves (subtracted mean percentage of autogenic losses) in dry weight (DW) per g of body weight (FW) per day. The egestion rate was calculated as the total mg of produced feces (DW) per g of body weight (DW), per day. The assimilation rate was calculated as the total mg of ingested leaves (DW) minus the total mg of produced feces (DW) per g of body weight (FW) per day (DW = dry weight; FW = fresh weight) (Loureiro et al. 2006).

Time of passage through gut and digestibility

We also measured the amount of time that the food was retained in the gut. Animals were kept in individual units containing carrot as a food source (generates fecal pellets that differ in color) for a week. Then, 10 animals were exposed to one disc of leaf litter for each decomposition stage, and monitored every two hours for 80 hours. We recorded the timing of the first sign of leaf consumption (i.e., evidence of nibbling on the leaf disc) and that of the first non-carrot feces appearance.

Digestibility was calculated using animals fed carrots for one week and placed into units containing one leaf disc and monitored daily until total consumption. Following full disc consumption, animals were fed carrots to maintain egestion of the leaf material from the gut. Feces from leaf feedings were collected, oven dried, and reweighted. Five units from each decomposition stage were used. The digestibility was calculated

as a percentage based on the total mg of ingested leaf minus the total mg of feces produced per mg of ingested leaf. Given the variability in duration with this method, other feeding rates were not calculated.

Phenolic and flavonoid content

The total phenolic content was determined using the Folin-Ciocalteau method (Bärlocher and Graça 2005) with tannic acid as standard. For each stage of decomposition, total phenolics were extracted from four samples of approximately 100 mg of ground up dry leaves in 5 mL of acetone for 1 hour at 4°C for a total of 20 samples.

Five samples of each decomposition stage were used to determine the flavonoid content. For each sample, five discs of dry leaves (or an approximate amount) were ground up and left for two days in 5 mL of ethanol 80% for flavonoid extraction. Flavonoid content was determined using the method reported by Zhishen et al. (1999) with modifications using quercetin as standard.

The mean concentrations of phenolics and flavonoids were multiplied by the consumption rates to estimate the total ingested amount of each group of substances.

Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test. The consumption, egestion, and assimilation rates were compared using a one-way analysis of variance (ANOVA) followed by Tukey's test. Pearson correlations were used to verify the association between consumption and egestion rates among treatments. All statistical analyses were performed using InStat 3.01 software.

Results

Feeding preference

The consumption rate was significantly higher when animals fed on *S. terebinthifolius* (52.9 ± 9.0 mg g^{-1} day^{-1},n = 10; mean ± SE) ($F_{2,26}$ = 9.395; $p < 0.001$), and no significant difference was recorded when animals fed on *L. brasiliensis* (31.8 ± 4.0 mg g^{-1} day^{-1}, n = 10) and *R. communis* (15.5 ± 2.9 mg g^{-1} day^{-1}, n = 9). The egestion and assimilation rates were 46.3 ± 9.8 (mg g^{-1} day^{-1}) and 11.2 ± 2.0 (mg g^{-1} day^{-1}) for *S. terebinthifolius*, and 21.1 ± 2.6 and 14.8 ± 1.62 (mg g^{-1} day-1) for *L. brasiliensis*, respectively. Egestion and assimilation rates could not be calculated for *R. communis* due to a low amount of fecal pellets. The phenolic content was highest in *L. brasiliensis* (71.1 mg of tannic acid equivalent per g of dry leaf), followed by *R. communis* (60.9), and was lowest in *S. terebinthifolius* (30.0) (Fig. 1). Standard error could not be calculated due to insufficient leaf material for additional rep-

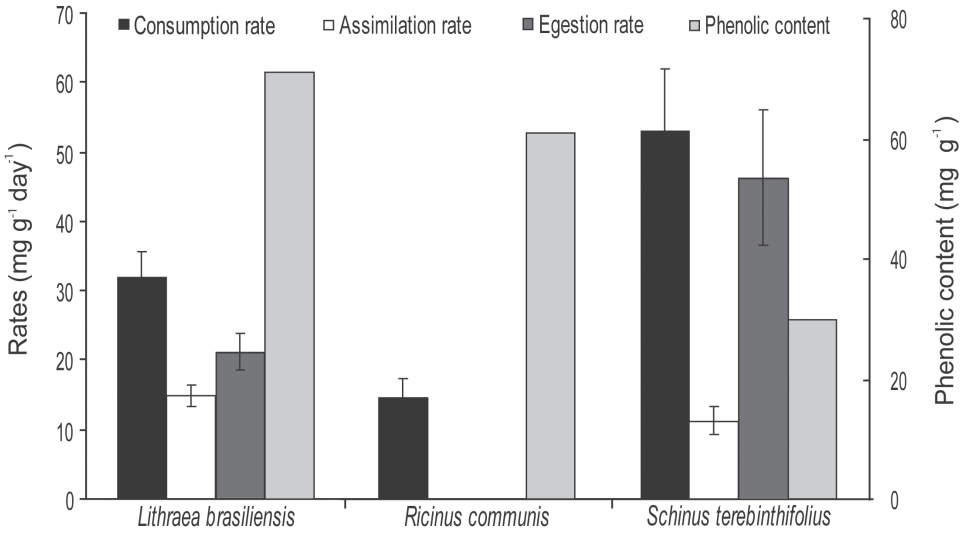

Figure 1. Isopod feeding rates on leaves of *Lithraea brasiliensis* (n = 10), *Ricinus communis* (n = 9), and *Schinus terebinthifolius* (n = 10) with 14 days of decomposition and respective phenolic content (standard error was not calculated due to the low amount of leaf remains for chemical analysis). Egestion and assimilation rate could not be calculated for *Ricinus communis* (low amount of fecal pellets). The values are mean and SE. Superscript letters indicate significant difference among treatments ($p < 0.05$).

licates. The mass loss of leaves in control units was 0.08% for *S. terebinthifolius*, 0.09% for *L. brasiliensis,* and 0.034% for *R. communis*. Mortality was 20% or lower in all treatments.

Feeding rates on leaves in different stages of decomposition

Schinus terebinthifolius was used to examine the feeding rates at different decomposition stages. The consumption rate was significantly higher on two month-old leaves ($F_{3,58} = 8.96$; $p < 0.0001$), and there were no significant differences between green, one-month-old, and three month-old leaves. The egestion rate was significantly higher for two-month-old leaves ($F_{3,58} = 14.17$; $p < 0.0001$) and there was no significant difference between green and one-month-old leaves, or between one-month-old and three-month-old leaves. The assimilation rate of green leaves was significantly higher than that of two- and three-month-old leaves and exhibited no significant difference between one-, two-, and three-month-old leaves ($F_{3,58} = 5.275$; $p = 0.0028$) (Table 1). The mean reduction in leaf mass in the control units was 0.16% (green), 0.13% (one-month-old), 0.12 (two-month-old), and 0.06% (three-month-old). Mortality was 0.35% for green, 0.15% for one-month-old, and 0.20% for two- and three-month-old leaves.

There were significant correlations between consumption and egestion rates for all decomposition stages. The correlation was stronger for one-month-old leaves ($r^2 = 0.928$; $p < 0.0001$) followed by those for three-month-old leaves ($r^2 = 0.9258$;

Table 1. Feeding rates of *Balloniscus sellowii* on *Schinus terebinthifolius* for different stages of decomposition. Data are expressed as mean value and SE of mg of food source (DW), per g of animal (FW), per day. N differs among decomposition stages due to different mortality in treatments. Different letters indicate significant differences of each rate among treatments ($p < 0.05$).

Decomposition stage	Consumption rate	Egestion rate	Assimilation rate
Green leaves (n=13)	41.5 ± 5.1 [a]	10.7 ± 3.2 [a]	30.7 ± 2.9 [a]
1 month-old leaves (n=17)	52.2 ± 4.6 [a]	29.1 ± 5.7 [a,b]	23.0 ± 1.7 [a,b]
2 months-old leaves (n=16)	80.1 ± 6.2 [b]	61.3 ± 6.4 [c]	18.9 ± 2.6 [b]
3 months-old leaves (n=16)	53.4 ± 5.5 [a]	33.4 ± 5.1 [b]	20.0 ± 1.5 [b]

$p < 0.0001$), two-month-old leaves ($r^2 = 0.8342$; $p < 0.0001$), and green leaves ($r^2 = 0.7240$; $p < 0.0002$).

Time of passage through gut and digestibility

The time required for passage through the gut did not differ among stages of decomposition. For all stages, the mode time for the appearance of leaf-based feces was two hours (one observation interval). However, leaf ingestion was initiated immediately for most units for the one-, two-, and three-month-old leaves, whereas the ingestion of the green leaves was initiated two days after the onset of the experiment. For 3 out of the 10 units containing green leaves, no apparent consumption had occurred after 80 h of observation.

The digestibility, calculated based on the total consumption of a leaf disc with a known mass and a second food source to push leaf material out of the gut, was 43.1 ± 3.8% for green leaves (n = 6), 19.7 ± 2.4% for one-month-old leaves (n = 4), 20.3 ± 1.6 for two-month-old leaves (n = 5) and 19.5 ± 2.8% for three-month-old leaves (n = 2).

Phenolic and flavonoid content

The phenolic content was significantly different across all decomposition stages ($F_{3,12} = 602.61$; $p < 0.0001$), and was highest in green leaves (66.0 ± 0.3 mg of tannic acid equivalent per g of dry leaf) and lowest in two-month-old leaves (36.9 ± 0.4 mg g^{-1}). The flavonoid content was significantly highest in green leaves (21.6±1.9 mg g^{-1}), but did not differ significantly among the other stages ($F_{3,16} = 37.10$; $p < 0.0001$). The estimated phenolic amount ingested by animals was not significantly different ($F_{3,58} = 2.065$; $p = 0.115$), ranging from 2.141 ± 0.190 mg of phenolic per g of isopod per day (one-month-old) to 2.954 ± 0.229 (two-month-old). The estimated flavonoid amount ingested by the animals followed the same trend as the assimilation rate among decomposition stages, being significantly higher in green leaves, but not differing between those undergoing one, two, and three months of decomposition ($F_{3,58} = 10.783$; $p < 0.0001$) (Fig. 2).

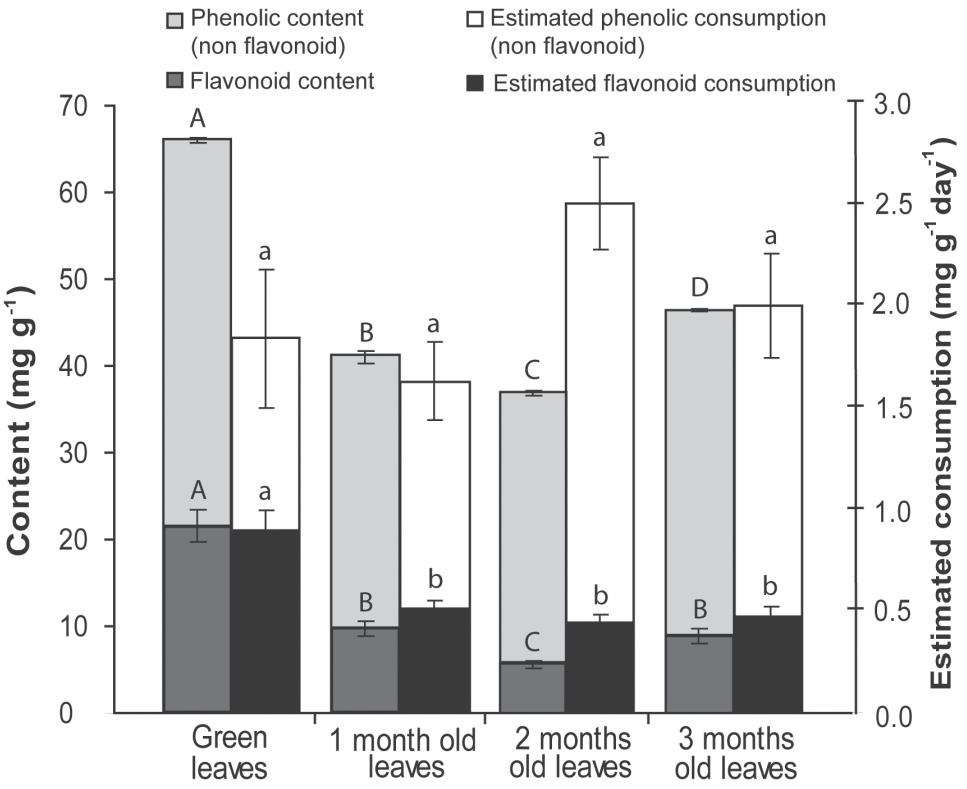

Figure 2. Total phenolic and flavonoid content and estimated amount of total phenolics and flavonoids ingested by *Balloniscus sellowii* on leaves of *Schinus terebinthifolius* for different stages of decomposition. The values are mg of equivalent of quercetin (flavonoid) or tannic acid (phenolic) per mg of dry leaf ± SE. Superscript letters indicate significant differences among treatments ($p < 0.05$).

Given that the flavonoid content was not tested for every experimental unit, the average content of each leaf age was correlated with the average assimilation rate for each stage of decomposition, resulting in a high correlation ($r^2 = 0.9688$; $p = 0.0157$, n = 4).

Discussion

Numerous studies have analyzed the effects of secondary metabolites in herbivores, whereas few studies have been conducted to understand the role of these compounds in detritivore and decomposer organisms. For example, an understanding of the presence of unpalatable or indigestible compounds and their rates of consumption related to leaf senescence is lacking. Our source site harbored plant species characteristic of a successional stage that do not exhibit mechanical structures to deter herbivores other than lignin, suggesting that chemical defenses are key for plant protection. Tropical plants inhabiting resource-poor environments invest heavily in chemical defenses such

as phenolics (Agrawal 2006). *Ricinus communis* exhibited the lowest consumption rate and constituted a small percentage of the fecal pellets egested. This plant has been associated with large amounts of secondary metabolites, including gallic acid, quercetin, and rutin, which represent some of the major phenolic compounds responsible for the antioxidant activity of its dry leaves (Singh et al. 2009). Its decomposition after two weeks resulted in viscous leaves that would be avoided by animals in natural conditions. *Lithraea brasiliensis*, despite being readily found within leaf litter in Brazil, also exhibits a large amount of secondary metabolites (Correia et al. 2006), and its consumption by *B. sellowii* was significantly lower than that of *S. terebinthifolius* and did not differ when compared to *R. communis*.

Schinus terebinthifolius accounted for the highest consumption rate and it is known as a source of terpenoids, simple phenolic derivatives, and flavonols. Furthermore, the antioxidant activity of the extract derived from its aerial parts has been described in the literature (Velázquez et al. 2003). Leaf extracts contain triterpene acids (Campelo and Marsaioli 1974), and the ethanolic extract of the leaves is a source of simple phenolics, several flavonoids, xanthones, and free steroids (Lima et al. 2006). Once decomposition is initiated, leaves begin to curl and might be used by animals for shelter as well as for feeding.

When given a choice, animals avoid green leaves that provide high amounts of secondary compounds (Cameron and LaPoint 1978). Ingesting decayed leaves is a behavioral strategy used to cope with chemically defended food, as the total amount of ingested defensive chemicals is reduced. Animals may use this strategy to increase their tolerance to chemically defended food (Glendinning 2007). For example, Roy and Bergeron (1990) observed a small rodent cutting leaves from branches then waiting for decomposition to occur before consuming them. We observed that the consumption rate increased throughout decomposition until the second month, when the highest palatability was observed, after which consumption decreased again in the third month of decomposition. Here, feeding on green leaves was a forced laboratory situation used to provide a comparison at an initial stage, and the leaves were oven dried before being offered to animals. This may have changed the leaf properties (as opposed to freshly fallen leaves), thereby allowing feeding. In addition, the decomposed leaves used in this study were handpicked from the trees rather than naturally senescent. However, storm events in this area are frequent, which allow broken branches to undergo decomposition and become available to isopods without undergoing senescence.

In general, detritivores show low digestibility, although differences in the digestibilities of high and low quality foods remain debated. In our feeding rates experiment, green leaves presented very high digestibility values (~80%, data not shown) and there was no difference in the time required for leaf-based feces to appear, contrary to the hypotheses of other researchers (Souza et al. 1998, Loureiro et al. 2006). However, upon calculating the assimilation efficiency based on the total consumption and egestion of a leaf disc and a second food source to push previous food through the gut, the digestibility values for green leaves were lower than those for decomposed leaves. The passage rates of leaves at different decomposition stages did not differ significantly when animals were fed continuously, and leaf-based feces always appeared within the

same day that feeding was initiated. However, it is possible that the amount of food retained in the gut under starvation conditions differs with food quality. Therefore, using a second food source to empty the animal gut of previously ingested food makes the estimate of digestibility more accurate. The digestibility values were higher when animals consumed green leaves, which was expected given that digestibility should decrease as decomposition progresses (Rushton and Hassall 1983, Hassall and Rushton 1984) due to the lower content of metabolites in decomposed leaves, which makes them more palatable (Johnson and Feldon 2001). Leaves having undergone one, two, and three months of decomposition exhibited similar digestibility efficiencies and assimilation rates. The assimilation rate is calculated by day and is less affected by the food stored in the gut during starvation at the end of the experiment than the digestibility.

Although the consumption rate did not differ significantly between green and one-month-old leaves, the onset of feeding on green leaves was not immediate, whereas it was for the other stages, suggesting that the high phenolic and flavonoid concentrations of green leaves are feeding deterrents and therefore reduce leaf palatability. These phenolic substances are probably lost in the beginning of the decomposition process due to leaching. If substances that cause feeding deterrence and inhibit feeding are lost early in the leaf decomposition process, the leaves will be consumed more quickly by detritivores, and thus be returned to the soil to provide nutrients back to the trees. Therefore, this process may serve as an adaptive advantage to the plants. Indeed, Cameron and LaPoint (1978) observed rapid leaching of tannins, which was associated with food inhibition from litter bags in the first week of decomposition.

During leaf senescence, the total secondary metabolite content tends to diminish due to leaching (Kuiters and Sarink 1986). Phenolics and flavonoids in plants are largely related to defense against pathogens and herbivores (Dixon and Steele 1999, Boué and Raina 2003), and leaves presenting a lower content of total phenolics are less toxic to isopods. Hassall and Rushton (1984) predicted that less heavily defended species would reach optimal palatability earlier than the climax species, which usually present more secondary metabolites for defense. In our study, the highest consumption was observed in two-month-old leaves, when the total phenolic and flavonoid content was lowest, even though it is thought that the phenolic signature rather than the total phenolic content determines detritivore consumption (Zimmer et al. 2005). The total phenolic and flavonoid content in this experiment increased in the third month rather than decreasing. Leaching of substances also occurs in other materials in the litter, and might increase the content of a specific constituent originating from the litter itself (from absorption) or from the action of microorganisms.

Flavonoids represented a considerable portion of the total phenolics in *S. terebinthifolius* leaves. We observed a correlation between the flavonoid content and the assimilation rate, whereas no correlation was detected with the total phenolic content. Although the flavonoid content of the leaves differed among decomposition stages, as did the consumption rates, the estimated amounts of flavonoids consumed by the animals were almost the same for leaves after one, two, and three months of decomposition. Thus, given that a decrease in the consumption of high flavonoid leaves is

not supported due to the significantly higher ingestion of flavonoids in green leaves, it appears that the animals increased their consumption of low flavonoid leaves, therefore suggesting that they might use these flavonoids as a food parameter.

Existing reports attribute the presence of phenolics and flavonoids in plants to defense against pathogens and herbivores, while only a few studies suggest possible benefits for organisms ingesting these substances. For example, when examining herbivores, Johnson and Felton (2001) also observed lower consumption and digestibility of plants that were overexpressing phenolics, but no significant reduction for growth and no indications of oxidative stress as a causal factor, suggesting a beneficial antioxidant property for herbivores. This study showed similar results, suggesting that the isopods might also be using flavonoids as an antioxidant agent. Even though the use of flavonoids by herbivorous invertebrates is not well documented, our data suggest that isopods may also use and discriminate concentrations of flavonoids, given that they appeared to increase consumption to minimize their intake of leaves with lower flavonoid contents. It is generally assumed that there is a maximum concentration of feeding deterrents that isopods can tolerate; however, additional studies are needed to examine the minimum intake of substances that can be used as a food parameter.

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Size dependent differences in litter consumption of isopods: preliminary results

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Abstract

A series of experiments were applied to test how leaf orientation within microcosms affect consumption rates (Experiment 1), and to discover intra-specific differences in leaf litter consumption (Experiment 2) of the common isopod species *Porcellio scaber* and *Porcellionides pruinosus*. A standardised microcosm setup was developed for feeding experiments to maintain standard conditions. A constant amount of freshly fallen black poplar litter was provided to three distinct size class (small, medium, large) of woodlice. We measured litter consumption after a fortnight. We maintained appr. constant isopod biomass for all treatments, and equal densities within each size class. We hypothesized that different size classes differ in their litter consumption, therefore such differences should occur even within populations of the species. We also hypothesized a marked difference in consumption rates for different leaf orientation within microcosms. Our results showed size-specific consumption patterns for *P. scaber*: small adults showed the highest consumption rates (i.e. litter mass loss / isopod biomass) in high density microcosms, while medium-sized adults of lower densities ate the most litter in containers. Leaf orientation posed no significant effect on litter consumption.

Keywords

Porcellio scaber, *Porcellionides pruinosus*, microcosm experiment, litter consumption, leaf orientation

Introduction

Ecosystem processes, such as decomposition, are greatly influenced by the taxonomic and functional diversity of assemblages (Tilman et al. 1997). At the same time, natural

populations consist of phenotypically various individuals representing differences in traits such as sex, age and morphology. Such differences have significant ecological consequences, such as intra-specific niche divergence among sexes (Shine 1989) and cohorts (Polis 1984, Werner and Gilliam 1984). In spite of these facts, as Bolnick et al. (2003) have pointed out *„…the majority of articles on measuring species' niche width make no mention of the fact that individuals of the same species may use different resources…",* because ecologists consider individuals as interchangeable when creating models for species interactions (Bolnick et al. 2011).

Confirming the statements above, studies suggest significant intra-specific divergence in the diet of soil macro-arthropods through their isotopic signatures (Okuzaki et al. 2009, Pollierer et al. 2009). Recently, Semenyuk and Tiunov (2011) showed similar results on several millipede species, suggesting that millipedes may change diet with their age. As isotopic signature reflects the diet of an individual (De Niro and Epstein 1978), the above mentioned differences suggest measurable differences in food preferences within populations.

Here, we focus on intra-specific differences in food consumption of terrestrial isopod species. Woodlice are relatively long-lived invertebrates that belong to the same guild (soil- and litter-dwelling macro-decomposers) in every life stage: manca, juvenile, pre-adult, adult. Isopods are effective decomposers (e.g. Hassall et al. 1987), and have a potential role in habitat remediation, thus, ecological restoration (e.g. Loureiro et al. 2006, Snyder and Hendrix 2008).

Decomposition through isopods is a phenomenon rather frequently studied, particularly in laboratory experiments (e.g. Szlávecz 1993, Zimmer et al. 2005, Hättenschwiler and Bretscher 2001). However, the papers seldom take note on the used age or size of animals as factors which may affect decomposition.

In our experiments we focused on distinct size classes of isopods. Intra-population variation in body size (especially between medium-sized and large adults) may also be explained by distinct life history patterns. As such, cohort-splitting is a phenomenon by which some individuals of a certain cohort grow slowly while others grow faster. Young males and females may grow differently, e.g. invest more in growth than reproduction in the early periods (e.g. Grundy and Sutton 1989).

Distinct isopod size classes, whether they are related to true age differences or cohort-splitting, may also differ in leaf litter utilization. This assumption is based on the idea that different size classes probably show differences in their feeding preferences – due to their anatomical features, e.g. ontogenic development in mouth parts, mandible morphology (Jackson 1928, Schmalfuss 2008) – on a given leaf (soft tissues vs. hard veins). Wieser (1966) has shown a size dependent, two-phase function in assimilation at the isopod *Porcellio scaber* Latreille, 1804. The referred work, however, made distinction between very small larvae (up to 0.3 mm) and larger individuals.

We conducted preliminary observations to detect how leaf orientation affects decomposition rates. We assumed that structural differences of the abaxial and adaxial sides of leaves will show distinctions in decomposition patterns. Isopods aggregate under logs and leaf litter to hide, mate and feed (Sutton 1980). Such shelters often serve

as food source as well. Leaves of most higher plants show dorsoventral differences in their adaxial and abaxial conformation representing characteristic structures and functions. For example, the epidermis and plant cuticle are thicker on the adaxial side while most stomata are located on the abaxial side. Given its relatively better accessibility, decomposition is more likely to affect the abaxial side first. This assumption, combined with the sheltering behaviour of isopods may lead to a greater mass loss in leaves placed with the abaxial side downwards.

Choosing species-poor ecosystems (e.g. urban areas) as model for our studies, we aimed to see how different size classes of frequent urban species, *P. scaber* and *Porcellionides pruinosus* Brandt, 1833, contribute to litter mass loss in laboratory experiments. For *P. scaber* we used two different sets of experiments applying different densities for each size classes.

In this paper we also present a detailed description of microcosm setup and experimental design. We added some new features such as a multi-layer plaster system to maintain constant humidity, and standard order of leaf orientation to avoid biases from selective feeding.

Hypotheses

A pilot study showed differences in litter degradation patterns among size classes of *P. scaber*: small bodied pre-adults primarily fed on leaf tissues amongst small veins, leaving vascular tissues and plant cuticle intact. Medium-sized adult individuals ate smaller veins and tissues, while large adults made no visible distinction in their choice (Fig. 1). Based on that observation, we assumed differences in litter consumption, too.

First, we hypothesize that leaf orientation affects consumption rates, explained by the general differences between abaxial and adaxial sides of tree leaves. We assume greater mass loss of leaves with abaxial side exposed, as abaxial side has thinner plant cuticle and protective layers (wax, hairs) than adaxial side.

Secondly, we hypothesized larger adults to consume more poplar litter due to their seemingly wider spectrum of food sources compared to the smaller ones.

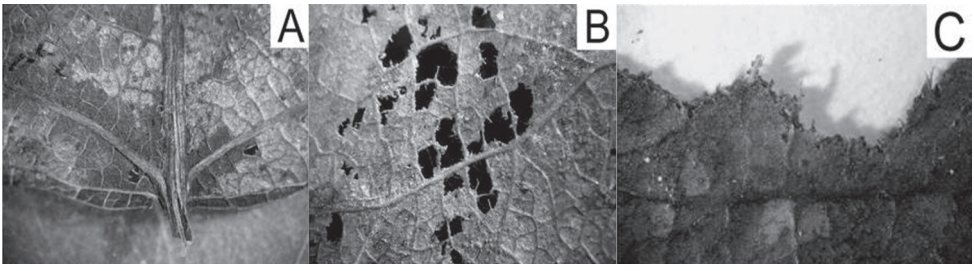

Figure 1. Patterns of litter degradation by *Porcellio scaber* size classes on poplar litter. A=small (0.2-0.5 mm in length), B=medium (0.7 – 10 mm), C=large (10 – 15 mm) isopods.

Methods

To test our two hypotheses, two separate microcosm experiments were set up. We established controlled conditions at the Institute for Biology at Szent István University, Budapest. To maintain constant humidity and temperature, we built a tent out of transparent plastic sheets and a wooden frame (5 m in length, 3 m in width, 2 m in height). The inner mean temperature was 19 ºC (SD±0.7), average relative air humidity was 68% (min. 61%, max 72%). We set a light regime of 12 / 12 hours of night and day.

Microcosms

For the experiments we used a set of microcosms assembled in the same way (Fig. 2). We used transparent poly-ethylene containers of 15 cm in height and 15 cm in diameter, with removable lids perforated for air exchange. To maintain constant humidity, we applied layers of plaster of Paris.

Solid plaster has already proven to be useful in laboratory experiments (e.g. Hassall et al. 1987, van Vliet et al. 1993): its water holding capacity maintains a humid environment in the container without making it too moist. To avoid water film on the surface (and e.g. fungal infection) we watered the containers from the bottom through additional layers of plaster of Paris. This system provided suitable humidity without getting too wet.

The humidity system consisted of three layers of plaster of Paris: a 3 cm thick bottom layer, a 0.5 cm bridging layer and a 1 cm thick contact layer. Plastic trays (bottom layer), shallow cups (bridging layer) and the containers for microcosm (contact layer) served as mold casting (see Fig. 2 for details).

The bottom layer was a slab of 40 cm x 30 cm plaster molded in a plastic tray watered directly. A hole was carved from one corner to monitor water level. As this layer should be always loaded with water, refill was necessary every second days (ca. 1.5 L / tray). As water does not always distribute evenly within plaster, it is important to maintain even surfaces.

The bridging layer, a thin disc of plaster, was placed between the watery bottom layer and the contact layer within the container. This layer was attached to the bottom of each container and transferred water to the contact layer through a hole cut on the bottom of each container. The bridging layer was glued to the contact layer by plaster. The contact layers, molded from microcosm containers, therefore precisely fit, were the solid grounds for isopods where litter consumption took place.

As preliminary studies showed a tendency for cannibalism (approx. 5% mortality), we decided to apply inedible shelters. For this purpose we used non-translucent rubber tubes (2 cm long, 0.5 cm diameter), 3 pieces in each container. Microcosms were placed randomly on trays of bottom layers.

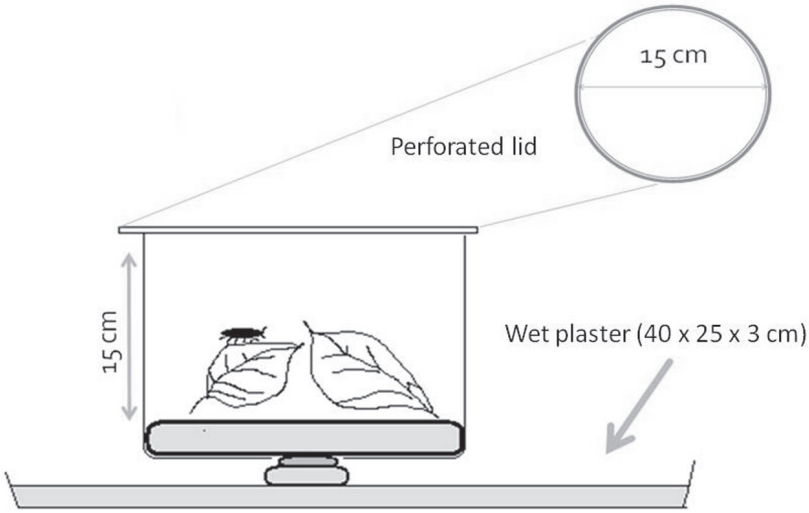

Figure 2. Schematic figure of a microcosm showing dimensions of the transparent plastic container and the plaster layer underneath.

Leaf litter

For both experiments we used leaf litter of *Populus nigra* L. collected one week after fall and stored in the lab under dry conditions. The chosen tree species is common in Hungarian urban green spaces and floodplain forests. Leaf litter was collected from a major park (Városliget) in Budapest. We used leaves of similar stage of decay: surface and edge were unbroken, the colour was brown. After washing off the dust, we removed the petioles from the leaves. Litter was oven dried at 60°C for 20 hours. Litter processing varied between experiments as described in sub-chapters below.

Test species

We chose the common rough woodlouse *P. scaber* and *P. pruinosus* as test species for our experiments. *Porcellio scaber* is a frequently used organism of laboratory research (e.g. Farkas et al. 1996, Ihnen and Zimmer 2008). Both species are frequent in urban environments and occur all over the world (Schmalfuss 2003).

Porcellio scaber individuals were collected in an enclosed garden in Hajdúböszörmény for Experiment 1, and in central Budapest for Experiment 2. Individuals of *P. pruinosus* were collected from a compost heap in the park of the Faculty of Veterinary Sciences, Szent István University, Budapest.

As isopods gut content may differ, we attempted to let the animals empty their guts without refilling it. All animals were starved for two days and weighed before and after the experiment with a digital analytical scale.

It is known that a single population may produce great annual, or even seasonal variations (e.g. Stearns 1992) in its metabolism. As all of our experiments were conducted in the similar period of the year (winter and early spring) period, under controlled (temperature, humidity, photoperiod) conditions, we consider our data comparable.

Data analyses

To test our hypotheses on leaf litter consumption we used Mood's median test as implemented in R (R Development Core Team 2012). The null-hypothesis of this robust, non-parametric test assumes that the medians of two data sets are not different. To calculate leaf litter consumption rates we divided litter mass loss (mg) by the average of the initial and final weights of isopods per microcosm. With this method we got the litter mass loss milligram per isopod milligram. Data of microcosms with a mortality higher than 20% were omitted.

Experiment 1: Effects of leaf orientation on litter consumption

To reveal the effects of different leaf orientation (adaxial side up or down) in litter consumption experiments, we placed standard discs (ca 2 cm in diameter) cut from *P. nigra* leaves in microcosms (c.f. Loureiro et al. 2006). With 13 replicates we used leaf discs with their abaxial side up, while in another 13 containers abaxial side down. Dry weights were measured after five days and compared to initial oven-dried dry weights for each container. As mortality in each container occurred in less than 20% of individuals, we included data from all containers to the analyses.

To the experiment either five *P. scaber* (size >1 cm, 238 mg ± 5 mg) or five *P. pruinosus* (cca. 0.5 cm, 71 mg ± 2 mg) adult individuals were used per container.

Experiment 2: Intra-specific, size dependent litter consumption rates

To each microcosm we added 1500 mg (± 1 mg) of poplar leaves, three pieces per container. As we supposed that litter orientation biases consumption rates, we arranged leaves in a standard order: 1st leaf abaxial side down, 2nd leaf adaxial side down, 3rd leaf abaxial side down, etc.

Litter dry mass was measured by a digital analytical scale at the start, and after 14 days of experimental period (with 12 hours light-dark regime). Faecal pellets and dirt were brushed off prior to final weighing after oven-drying (60 °C) for 20h.

Out of the collected individuals we selected three distinct size classes (small: 3-5 mm, medium: 7-11 mm, large: >15 mm).

Within Experiment 2, we used two experimental settings by using different isopod densities within microcosms. We attempted to use similar numbers of isopod individuals in similar weights at each size class per each replicate (microcosm). Mean numbers, biomasses and the number of microcosms used in our experiments are shown in Table 1.

At Experiment 2/a we attempted to keep isopod biomass constant at around 200 mg in each microcosm, regardless to size classes. Whereas in Experiment 2/b we used only *P. scaber* individuals in densities higher than in Exp. 2/a. In this case, isopod biomass was kept at around 300 mg in each microcosm for all size classes.

Data of containers with a mortality rate higher than 20% were omitted from analyses. In order to keep similar sample sizes, we had to omit data from other size classes as well (even if their mortality rate was lower than 20%). In such cases we used a random number generator by which we selected data for deletion. This practice has also resulted in differences in numbers of replicates among Experiments, as seen on Table 1.

Table 1. Mean (±SD) number of isopod individuals, their cumulated biomasses and the number of microcosms used in the experiments.

Size category		Experiment 2/a				Experiment 2/b	
		Porcellionides pruinosus		*Porcellio scaber*		*Porcellio scaber*	
Small	No. of ind.	20	(SD±0)	12	(SD±0)	51	(SD±5.9)
	Biomass (mg)	82.5	(SD±1.87)	224.3	(SD±14.76)	301.1	(SD±2.76)
Medium	No. of ind.	10	(SD±0)	6	(SD±0)	8.9	(SD±0.76)
	Biomass (mg)	85	(SD±3.35)	166	(SD±2.74)	303.7	(SD±8.67)
Large	No. of ind.	5.2	(SD±0.24)	3	(SD±0)	4.7	(SD±0.23)
	Biomass (mg)	83.5	(SD±2.26)	232.8	(SD±11.29)	299.9	(SD±2.89)
Number of microcosms analysed		8		9		10	

Legend: No. of ind. = Number of individuals within microcosms; Biomass = isopod biomass (mean±SD) within microcosms

Results

Experiment 1: Effects of leaf orientation on litter consumption

With *P. scaber* we found visible, albeit not significant (Mood's median test, p=0.11), differences in litter mass loss (Fig. 3). Similarly, Mood´s median test revealed no significant effect of leaf orientation on the litter consumption rates of *P. pruinosus* (p=1).

Experiment 2/a and 2/b: Intra-specific differences in litter consumption

Litter mass loss differed among the three size classes of *P. scaber* at both experimental sets (2/a,b), while no significant differences were found among classes of *P. pruinosus.* Figure 4 shows the main results: A and B represent Experiment 2/a, while C represents Experiment 2/b.

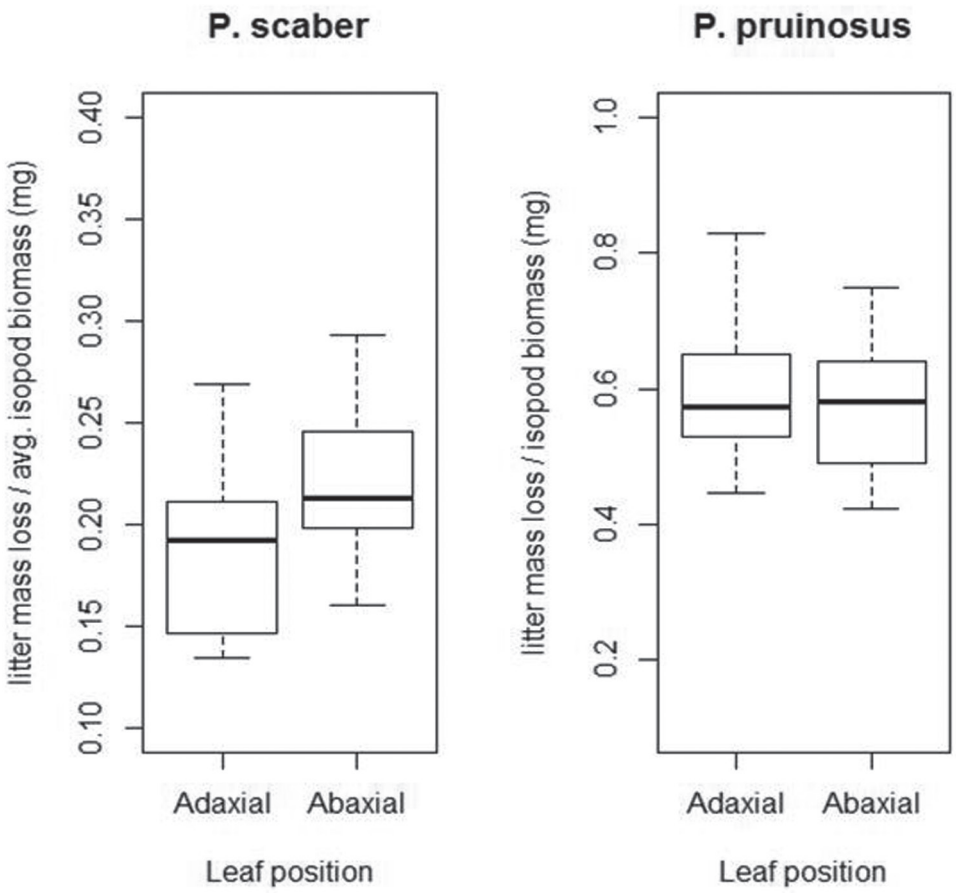

Figure 3. Effects of leaf litter positioning (abaxial up vs. abaxial down) on litter consumption of two isopod species. Note the different scaling of y axes. Legend: P. pruinosus: *Porcellionides pruinosus*; P. scaber: *Porcellio scaber*; Adaxial = adaxial side up; Abaxial = abaxial side up; Thick line = median; box = lower and upper quartiles; whisker = min. and max values.

In Experiment 2/a (lower *P. scaber* densities) we measured statistically significant differences between „medium" and both „large" and „small" classes (Fig. 4/A). The latter two categories showed no statistically significant difference in their litter consumption, (p=0.131). *Porcellionides pruinosus* size classes showed no marked difference in their litter consumption (p=1).

In Experiment 2/b (the setting with higher *P. scaber* densities) we measured significant differences between the „small" and both „medium" and „large" classes (Mood's median test, p<0.001), while the two larger categories showed no significant difference (p=1).

All *P. pruinosus* size classes consumed, in general, ca. 1 mg_{leaf} / 1 mg_{isopod}, while this rate was less in most *P. scaber* size classes in both experimental settings (Fig. 4).

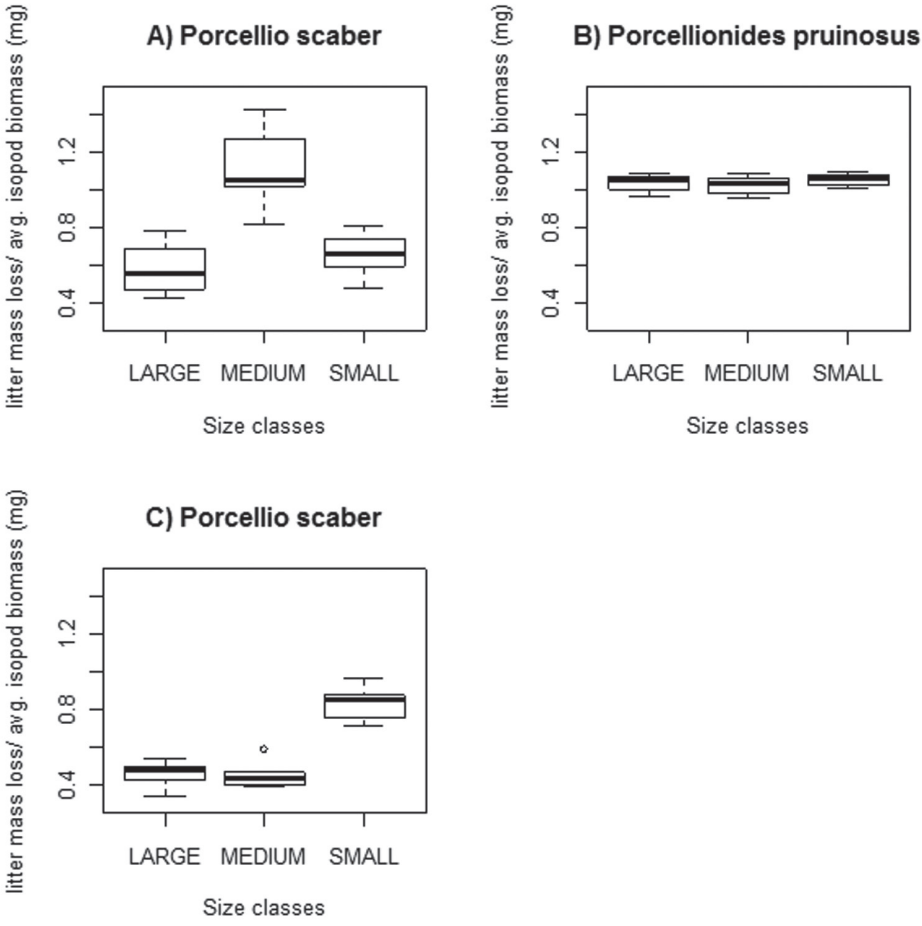

Figure 4. Freshly fallen poplar litter consumption of three size classes of *Porcellio scaber* and *Porcellionides pruinosus*. Legend: Thick line = median; box = lower and upper quartiles; whisker= min. and max values; open circles: outliers.

Discussion

The study proved that (with the current setup) leaf orientation do not have significant effect on leaf litter consumptions. Isopod size classes, to certain degrees however, can bias leaf litter consumption rates in microcosm experiments.

It is evident that at this stage of our research we are unable to explain the reasons of the patterns we got. Therefore we devote most of this chapter to speculations.

Leaf orientation, which had - to the best of our knowledge - never been studied before, has proven to pose no effect in biasing consumption rates. Our approach applies for microcosm experiments using small number of leaves (cc. 1–3) where „random" arrangement is not possible. We assume that greater differences would appear in consumption rates between the two sides as soon as isopods could reach

the bottom of leaf litter. Woodlice normally hide under dead plant matter, using it as shelter and food source at the same time (Stachursky 1968). The top of a leaf may be exposed to predators and other danger, so feeding on the surface may not be natural for isopods.

Leaves exposed to sun are large and thin while small and thick leaves develop in the shade (e.g. Jackson 1967). Sun leaves have a more developed spongy and palisade mesophyll regions, and higher photosynthetic rates in comparison to the shadow leaves on the same tree (e.g. Nobel 1976). Several studies prove that solar radiation activates flavonoid biosynthesis resulting sun leaves to contain higher amounts of phenolics (e.g. Jaakola et al. 2004). At the same time, litter quality differs between urban and rural habitats (McDonnell et al. 1997). These facts suggest that selecting the right leaves may be of great importance, as well.

Opposite to our hypothesis, large *P. scaber* individuals ate relatively less than smaller adults. In fact, large adults consumed very little of the leaf litter. For this reason we suspect that the primary food source of this species may be something more palatable than freshly fallen (or near-freshly fallen) litter. The relatively large consumption rates for smaller (small- and medium-sized) adults is probably explained by their higher metabolism induced by intense growth. This agrees with the findings of Reichle (1968) who showed an inverse correlation between metabolic rates and live body weights for different arthropod species (i.e. smaller arthropod species had higher metabolic rates). Pennington and Meehan (2007) have, however, shown that metabolic rates of centipedes had a positive correlation with body mass. Still, as intra-population variations of the metabolic rates of other Porcellionid species (*Porcellio laevis* Latreille, 1804) is also known (Lardies and Bozinovic 2008), we should regard that phenomenon as of high importance in understanding patterns of intra-population litter consumption by woodlice. Still, the reason why *P. pruinosus* size classes displayed nearly equal consumption patterns remains unanswered.

Our results with *P. scaber* suggest that mainly the small individuals contribute in the comminution process of leaf litter. This function may be especially valuable in areas with low soil activity and species poor decomposer fauna, such as urban areas (e.g. McDonnell et al. 1997). Size dependent litter mass loss and functional differentiation within populations may be estimated by combining seasonal activity and demography data of natural populations with in-vitro litter mass loss rates.

Besides the effects of size classes, we have also shown results more likely related to densities (individuals per container) than isopod sizes (Rushton and Hassall 1987). Based on our results, we suppose that density poses a substantial effect on litter mass loss in microcosm experiments. On the analogy to the Allee-effect (e.g. Stephens et al. 1999), we think that litter mass loss increases with an increasing density until it reaches a point where intra-population competition stabilizes or even decreases consumption rates.

Conclusions

With these results, we would like to show some details that can, to some degree, bias results in laboratory experiments. In order to provide more reliable results in microcosm experiments we suggest standardizing the size and density of isopods.

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The authors thank Dr. Péter Szabó and Dávid Fülöp for their valuable contributions to practical and theoretical challenges during the experiments. Our project was funded by NKB ÁOTK 15942. The authors would like to thank the Referees for their detailed review and helpful comments.

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Prolonged feeding of terrestrial isopod (*Porcellio scaber*, Isopoda, Crustacea) on TiO_2 nanoparicles. Absence of toxic effect

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Abstract

Nanoparticles of titanium dioxide are one of most widely used nanomaterials in different products in everyday use and in industry, but very little is known about their effects on non- target cells and tissues. Terrestrial isopods were exposed to food dosed with nano-TiO_2 to give final nominal concentration 1000 and 2000 μg TiO_2/g dry weight of food. The effects of ingested nano-TiO_2 on the model invertebrate Porcellio scaber (Isopoda, Crustacea) after short-term (3 and 7 days) and prolonged (14 and 28 days) dietary exposure was assessed by conventional toxicity measures such as feeding rate, weight change and mortality. Cell membrane destabilization was also investigated. No severe toxicity effects were observed after 3, 7, 14 or 28 days of dietary exposure to nano-TiO_2, but some animals, particularly those exposed to lower concentrations of nanoparticles, had severely destabilized digestive cell membranes. It was concluded that strong destabilization of the cell membrane was sporadic, and neither concentration- nor time-related. Further research is needed to confirm this sporadic toxic effect of nanoparticles.

Keywords

Isopods, *Porcellio scaber,* TiO_2 nanoparticles, prolonged feeding, toxic effects

Introduction

During the last decade the presence of nanomaterials has increased extraordinarily, and information on their toxicity is urgently needed. Nanomaterials have unique physical and chemical properties as a result of their small particle size, shape, conductivity and surface characteristics. TiO_2 nanoparticles are most commonly encountered nanoparticles and as a consequence they could become a substantial environmental pollutant. Nanoparticles of TiO_2 have been shown to have different types of effects *in vivo* (Menard et al. 2011), although their toxic potential appears not to be very pronounced. Many studies indicate that the effects of nanoparticles differ significantly from those of soluble pollutants. There are some indications that nanoparticles may have some nanoparticle-specific effects on biological systems.

Many reports using terrestrial isopods as toxicity test organisms for chemicals and particles in laboratory single-species tests can be found in the literature, and *Porcellio scaber* (Isopoda, Crustacea) is among the most frequently used species in such studies. The species was found to be suitable in tests of the effects of elevated concentrations of metals (Drobne and Hopkin 1994, 1995, Jereb et al. 2003, Zidar et al. 2005), biocides (Staak et al. 1998, Stanek et al. 2006), veterinary drugs (Kolar et al. 2010, Zizek et al. 2011), and nanoparticles (Drobne et al. 2008, Jemec et al. 2008, Pipan-Tkalec et al. 2010, 2011). In these tests, several endpoints have been assessed, including biochemical biomarkers, histopathological changes, behavioral response and physiological measures and organism level responses. The selected biomarkers vary in their sensitivity.

Conventional measures of toxicity such as investigations of growth, reproduction, and life-cycle are not the most suitable when terrestrial isopods are the test organism. Rates of growth in terrestrial isopods over several weeks are variable even for a single individual (Van Capelleveen 1987); reproduction is difficult to assess because after mating, females may retain the sperm for a long period before reproducing, and the life cycle of most terrestrial isopods is relatively long, often more than 6 to 8 months (Drobne 1997).

In toxicity tests with isopods however, feeding parameters have proved to be an integrated organism-level response, appropriate evidence of the effects of chemicals. Feeding rate changes are relatively fast and have been observed in relation to added metals or organic chemicals. Reduced feeding rate in comparison to controls was recorded after exposure of isopods to metals and biocides (Drobne and Hopkin 1995, Drobne at al 2008) and in addition, measurements of feeding rate are non-invasive and feeding rates can be recorded both during and after the exposure (Drobne 1997) Finally, after exposure, many additional biomarkers at lower levels of biological complexity can be analyzed too.

Recently, studies on the effects of nanoparticles were performed with *P. scaber*. When added to food, TiO_2 particles had no adverse effect on the feeding rate of *P. scaber* after 3 or 14 days dietary exposure (Drobne et al. 2008) to up to 1000 μg/g dry food. In this study TiO_2 nanoparticles (nano-TiO_2) were reported even to enhance the feeding rate of *P. scaber*. Similarly, Jemec et al. (2008) reported no reduction in food consumption by *P. scaber* when feeding on nano-TiO_2 (3000 μg/g dry food) for 3 days. In a study

by Pipan-Tkalec et al. (2010), in which animals were exposed for 4 weeks to food dosed with 2000 or 5000 μg ZnO nanoparticles/g dry weight of food, the feeding rate was not affected by the elevated concentrations of Zn in the food and no adverse effect on feeding behavior was recorded after 14 days of exposure to silver nanoparticles up to 5000 μg nano-Ag/g dry weight of food (Pipan-Tkalec et al. 2011). These studies indicate that while feeding parameters are not affected within one or two weeks of exposure, they will be affected ultimately along with biomarkers at lower levels of biological complexity.

In the present study, the effects of nano-TiO_2 on the model invertebrate *Porcellio scaber* (Isopoda, Crustacea) after brief (3 and 7 days) and prolonged (14 and 28 days) dietary exposure are examined. We discuss the toxic effect of ingested TiO_2 nanoparticles on this terrestrial isopod. The feeding rate was used as evidence of a toxic effect and the cell membrane destabilization as a measure of a cytotoxic effect. We have found that after 14 days of exposure to nano-TiO_2, the feeding rate of *P. scaber* was not significaly affected, but cell membranes were destabilized in more than 40% of the population. If cell membrane destabilization leads to cytotoxicity, prolonged exposure of *P. scaber* to nano-TiO_2 will result in toxic effects which can be assessed by conventional toxicity measures. If there is a reduced feeding rate after prolonged exposure, this will confirm the time- and dose-dependency of the effects of nano-TiO_2 which has been seen with other materials.

Materials and methods

Chemicals

Acridine orange (AO), ethidium bromide (EB) and titanium dioxide nanoparticles (nano-TiO_2) were purchased from Sigma-Aldrich. The nano-TiO_2 was the same as was used in our earlier experiments (Valant et al. 2009) and was supplied as a powder, guaranteed 99.7% pure, with an anatase crystal structure, average particle size <25 nm and surface area between 200 and 220 m^2/g.

Model organisms

Terrestrial isopods (*Porcellio scaber*, Isopoda, Crustacea) were collected in July and August 2010 at location (46°4'20"N, 14°26'51"E) near Ljubljana, Slovenia. The animals were kept in a terrarium filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*), at a temperature of 20 ± 2°C and a 16:8-h light:dark photoperiod. Adult animals of both sexes, weighing more than 30 mg, were used in the experiments. If moulting or the presence of marsupia were observed at any time, the animals were removed from the experiment in order to keep the investigated population as physiologically homogenous as possible.

Characterization of nanoparticles

Nanoparticles were inspected with transmission electron microscopy (TEM), Brunauer-Emmett-Teller (BET) analysis, dynamic light scattering (DLS) and X-ray powder diffraction techniques. TEM micrographs were published in a previous report by Valant et al. (2009). Before and after the exposure of isopods to nano-TiO_2, three randomly selected pieces of leaves were dried, attached to mounts with silver paint (SPI), gold/palladium-sputtered (Sputter Coater SCD 050, BAL-TEC, Germany) and investigated at the Institute of Metals and Technology, Ljubljana, Slovenia with a field emission scanning electron microscope (FE-SEM; Jeol JSM-6500F). Energy dispersive X-ray analysis (EDX; EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F) was used to confirm the chemical composition of nanoparticles on the leaves.

In the DLS analysis, the dispersions of nanoparticles (100 μg nano-TiO_2/ml distilled water) were examined with a 3D DLS-SLS spectrometer (LS Instruments) which allows the assessment of the hydrodynamic radii of particles in extremely turbid suspensions by a 3D cross-correlation technique that eliminates light scattering. The light source used was a HeNe laser operating at a wavelength of 632.8 nm and scattering was measured at an angle of 90°. At higher concentrations of nanoparticles (1000, 2000 μg nano-TiO_2/ml distilled water), measurements were not possible, due to the low transparency of the samples (Valant et al. 2009). The same particles were also tested in some other studies which provided a more detailed description of their characteristics (Valant et al. 2009).

Experimental design

Hazelnut leaves were dried at room temperature, and cut into pieces weighing ~100 mg. The TiO_2 nanoparticles were suspended in distilled water to obtain different final concentrations (1000 and 2000 μg/ml). In a control group, the leaves were treated with pure distilled water. A suspension of particles was brushed onto the abaxial leaf surface and the leaf was allowed to dry, giving final nominal concentrations of nanoparticles on the leaves of 1000 and 2000 μg nano-TiO_2 per gram (dry wt) of leaf.

A single hazelnut leaf treated with either distilled water or nano-TiO_2 suspension was placed in a Petri dish with one animal in each Petri dish. The leaf was the only food source for animal. The Petri dishes were kept in a large glass container under controlled conditions in terms of humidity (≥80%), temperature (21±1°C) and light regime (16:8-h light:dark photoperiod). In experiments 1-4, animals were exposed for 3 days, 7 days, 14 and 28 days, respectively. During the 14 and 28 days exposure feces were removed every 7 days to eliminate the possibility of coprophagy. After the exposure the animals were weighed, and anasthetized and decapitated. The digestive

glands were isolated and used for assessment of digestive gland cell membrane stability as described below.

Feeding parameters, weight change and mortality

After 3, 7, 14 or 28 days of exposure of animals to treated leaves, fecal pellets and leaves were removed from the Petri dishes and the leaves were dried at room temperature for 24 h. The dried leaves and fresh animals were weighed and the feeding rate of the isopods was calculated as the mass of consumed leaf per animal's wet weight per day. The animal's weight-change in each case was defined as the change in animal wet weight from the beginning to the end of the experiment.

Digestive gland cell membrane stability

The AO/EB assay is based on the assumption that changes in cell membrane integrity result in differences in permeability of cells to AO and EB dyes. Different permeability to the two dyes results in differentially stained nuclei. Acridine orange is taken up by cells with membranes that are intact or destabilized, and in the cell, emits green fluorescence, as a result of its intercalation into double-stranded nucleic acids. Ethidium bromide on the other hand, is taken up only by cells with destabilized cell membranes, and it emits orange fluorescence, after intercalation into DNA (McGahon et al. 1995). Spectroscopy is used to assess the difference between green and orange emissions, and this provides a measure of cell membrane destabilization.

Cell membrane stability was tested with a modified method described by Valant et al. (2009). After isolation, one hepatopancreatic tube was incubated for 5 min in a mixture of acridine orange and ethidium bromide and then put on a microscope slide. Fresh samples were photographed and examined by an Axioimager.Z1 fluorescent microscope (Zeiss) with two different sets of filters. The excitation filter, 450 to 490 nm and the emission filter, 515 nm (filter set 09) were used to visualize AO and EB-stained nuclei, while the excitation filter, 365 nm and the emission filter, 397 nm (filter set 01) were used to visualize nuclei stained with EB alone. Cell membrane integrity was assessed by examination of micrographs. Photographs of intact digestive glands were examined by the same observer twice at intervals of at least 24 h. Cell membrane integrity was assessed visually and classified from 0 to 9 according to a scale of digestive gland cell membrane stability values predefined on the basis of preliminary experiments. This scale defines non-treated (control) animals as showing <5% of nuclei stained by EB, while severely stressed animals have up to 100% of EB-stained nuclei (Valant et al. 2009). In this study the <5% of hepatopancreatic tubes stained with EB were classified as 1 or 2, those with a medium proportion of stained nuclei 3 or 4 and those with the highest proportion (>95%) of EB-stained nuclei as 5 or 6.

Data analysis

The differences in the medians of measured parameters in exposed and unexposed groups were tested with the non-parametric Mann-Whitney U test. All calculations were done using Statgraphics Plus 4.0 statistics software. Statistical differences between exposed and control animals were categorized into three groups to which different numbers of asterisks were assigned (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Characterization of nanoparticles

Scanning electron microscopy revealed the distribution of TiO_2 particles applied on the lower leaf surface (Fig. 1a) and EDX confirmed their composition (Fig. 1b). The same particles were tested in other studies which provide a more detailed description of their characteristics (Valant et al. 2009). The DLS revealed the hydrodynamic radii of particles in the suspension applied to leaves as 110 nm. The BET method revealed that the surface area of TiO_2 samples was 144 m^2/g. The size and surface area correspond to the data provided by the supplier, and the X-ray powder diffraction data confirmed that the TiO_2 was in the anatase crystal form.

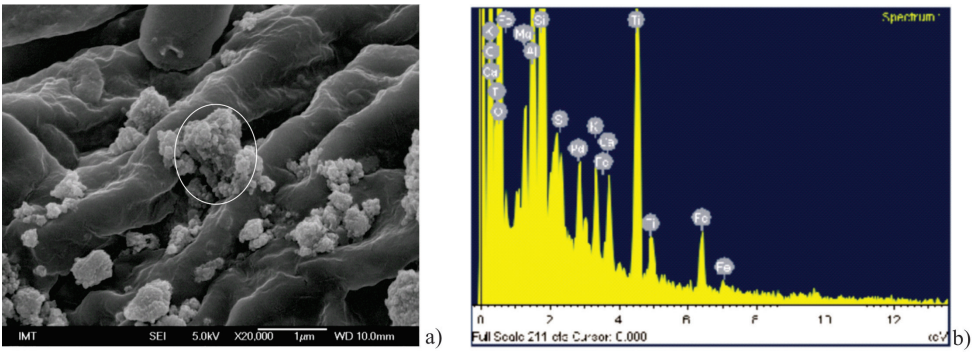

Figure 1a, b. TiO_2 nanoparticles dispersed over the abaxial leaf surface to give final concentration of 1000 μg/g dry wt of leaf **a** EDX spectrum of area encircled on Figure 1a, where presence of Ti is confirmed **b**.

Feeding parameters, weight change and mortality

The number of exposed animals at the beginning of the exposure and that at the end of the exposure failed to correspond because some animals molted during the course of experiment and consequently were excluded from further analysis. Based on the amount of consumed food it was estimated that when animals were fed on 1000 μg

nano-TiO_2/g of leaf they consumed approximately 0.01 ± 0.01 μg TiO_2 per day in 3 days, 0.05 ± 0.03 μg TiO_2 per day in 7 days, 0.07 ± 0.02 μg TiO_2 per day in 14 days and 0.05 ± 0.02 μg TiO_2 per day in 28 days. When fed on 2000 μg nano-TiO_2/g of leaf they consumed approximately 0.08 ± 0.04 μg TiO_2 per day in 3 days, 0.09 ± 0.03 μg TiO_2 per day in 7 days, 0.09 ± 0,03 μg TiO_2 per day in 14 days and 0.1 ± 0.05 μg TiO_2 per day in 28 days. No significant effect of ingested nano-TiO_2 on survival and weight change was observed in animals fed with TiO_2 nanoparticles when compared to control animals fed with untreated food.

There was a statistically significant decrease in feeding rate in animals exposed for 3 days or 14 days on food dosed with 2000 μg/g nano-TiO_2 when compared with controls (Fig. 2). However, an increase, also statistically significant, occurred in the feeding rate of animals exposed for 14 days to food dosed with 1000–μg/g nano-TiO_2 when compared with control ($p = 0.03$). These data indicate a dynamic response of feeding behavior to presence of particles in the food, which was not consistent over time (Fig. 2).

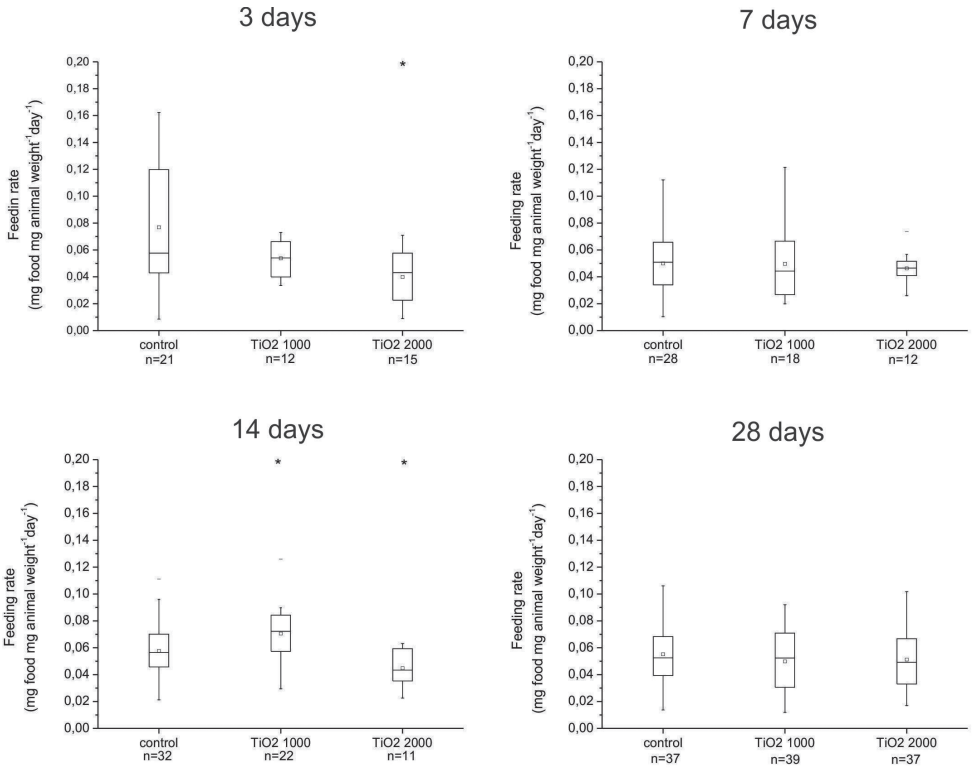

Figure 2. Daily feeding rate (mg of consumed leaves/animal weight) of animals fed on control (untreated) leaves and leaves dosed with 1000 or 2000 μg/g nano-TiO_2 for 3, 7, 14 or 28 days. On x scale also number of animals in each group is represented (n). There are statistically significant differences between animals exposed to food dosed with 1000 μg/g nano-TiO_2 for 3 and 14 days compare to control of the corresponding group and between control and 2000 μg/g nano-TiO_2 in animals exposed for 14 days (* $p < 0.05$). Symbols on the box plot represent minimum and maximum data values (whiskers), mean value (□), 75th percentile (upper edge of box), 25th percentile (lower edge of box), median (line in box) and max and min value (-).

In more or less all exposed groups the average feeding rate was similar, indicating reproducibility of the feeding parameters in different experiments.

Digestive gland cell membrane stability

Our previously published data demonstrate that in animals from a stock culture and in good physiological condition, the digestive gland cell membrane stability classification was higher than 2 in only 5% of animals, and this was considered to be a benchmark (Valant et al. 2009). The higher the value the more the membrane is destabilized.

Our data show that among the control animals fed with uncontaminated food, the digestive cell membranes were affected in up to 10% of exposed animals. We consider this to be a response to suboptimal experimental conditions in terms of isolation of animals, inappropriate shelter during the experiment, and poor food. We consider 10% of animals with affected cell membrane to be normal (benchmark).

The most significantly affected groups were those exposed to 1000 µg/g nano-TiO_2 for 3 days. In this group, 25% of animals had a cell membrane destabilization value of

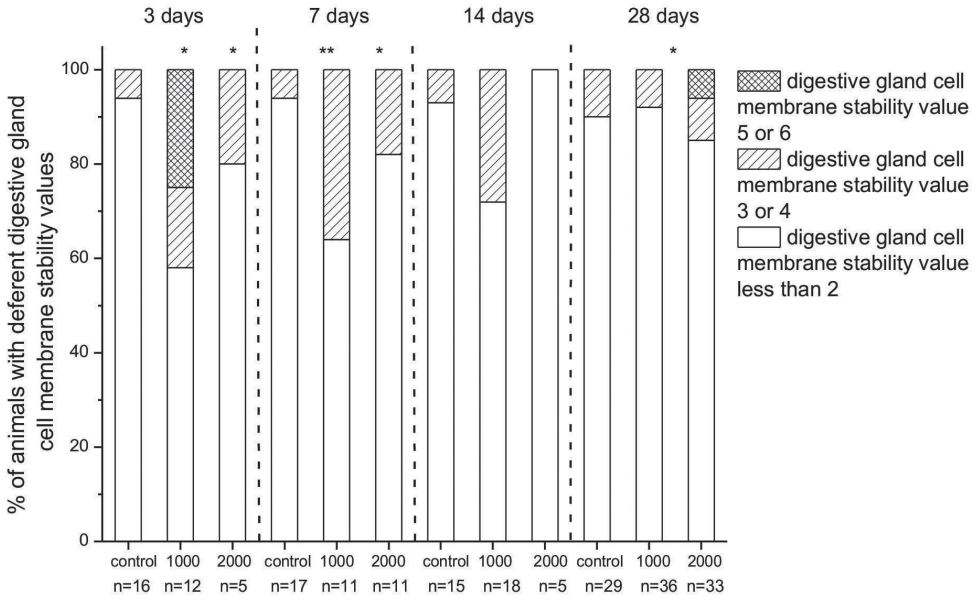

Figure 3. Percentage of animals in fed on food dosed with 1000 or 2000 µg/g nano-TiO_2 for 3, 7, 14 or 28 days with different degrees of destabilization of cell membranes, assessed visually and classified from 0 to 6 according to the scale defined in Materials and Methods, above. On x scale also number of animals in each group is represented (n). Digestive gland cell membrane stability values ≤ 2 represent animals which had no destabilized cell membrane and digestive gland cell membrane stability values 3 or 4 animals with destabilized cell membranes. Those with value 5 or 6 had the most destabilized cell membranes. Statistical differences between exposed and control animals (within one exposure duration) are marked with an asterisk (* $p < 0.05$ and ** $p < 0.01$).

5 or greater. After 7 and 14 days of exposure to food dosed with 1000 μg/g nano-TiO_2, digestive cell membrane destabilization was detected in 36% and 28%, respectively, of the exposed animals, but after 28 days of feeding on food dosed with 1000 μg/g nano-TiO_2 there was almost no effect (8%) on digestive gland cell stability. The highest concentration of TiO_2 particles in food (food dosed with 2000 μg/g nano-TiO_2) investigated was generally less harmful to digestive cell membranes, although in some (6%) of the animals exposed for 28 days, serious destabilization of the digestive cell membranes was observed (Fig. 3). These severe biological effects (after 3 and 28 days of exposure) were neither dose- nor duration-related and this cell membrane damage, which was never seen in control animals, could be interpreted as a sporadic effect.

Discussion

In our study no toxic effects could be confirmed by conventional toxicity parameters such as weight change or mortality in short-term (3 and 7 days) and prolonged (14 and 28 days) exposure. The changed feeding rate of exposed animals compared to controls is also convenient evidence of the effects of chemicals on isopods. We hypothesize that the adverse effect of chemicals is manifested in a reduced feeding rate. In cases where the feeding rate significantly increases, it is thought to be a hormetic like response (Drobne et al. 2008). Hormetic like response means that exposure to substances stimulate and not retard the measured response. This is a case when organisms are exposed to lower concentrations of substances and when effects at higher levels of biological complexity are recorded.

In this study, the feeding rate of the animals increased, decreased or was not affected at all. These observations coincide with our previous results in which nanosized TiO_2 enhanced the feeding rate of *P. scaber* (Drobne et al. 2008) while some other chemicals caused reduced feeding activity (Drobne and Hopkin 1995).

Different studies report changed feeding rates after feeding animals on chemically dosed food for different periods of time. For example, the feeding rate in *P. scaber* was assessed after 3 days (Drobne et al. 2008), 14 days (Drobne et al. 2008, Stanek et al. 2006), 21 days (Staak et al. 1998, Kolar et al. 2010), 28 days (Zidar et al. 2009, Zizek et al. 2011) and 35 days (Drobne and Hopkin 1995) of exposure to different substances. In juveniles, the feeding rate was statistically significantly reduced when animals were fed for 3 days with 50 μg/g of the pesticide imidacloprid, while in adults it was reduced when 10 μg/g of imidacloprid were incorporated in the diet (Drobne et al. 2008). No effect was found, however, on the feeding rate in *P. scaber* after exposure for 14 days to the pesticide diazinon (Stanek et al. 2006) at levels up to 100 μg/g. Staak et al. (1998) failed to observe any response after 21 days of exposure to the herbicide trifluralin. Kolar et al. (2010) reported that the antiparasitic veterinary drug abamectin (NOEC = 3 mg/kg dry soil) significantly reduced the food consumption rate in *P. scaber* at levels of 10 mg/kg in dry soil after 21 days exposure, but no effect was observed after 28 days exposure of isopods to the polyether antibiotic monensin

(NOEC ≥ 849 mg/kg dry soil, NOEC ≥ 357 mg/kg dry food) (Zizek et al. 2011). There are also reports of studies on the effects of metals (Zn, Cu, Co and Cd) on *P. scaber.* Zidar et al. (2009) reported that after 28 days of exposure to metals, a dose-related decrease in food consumption rate was observed, when a mixture of Zn and Cd was included in the food at nominal levels (2600 mg Zn + 360 mg Cd/kg dry food). The same group (Zidar et al. 2003) also documented a reduced feeding rate after exposure of the animals to 1800 μg Zn/g, 1200 μg Cu/g or 125 μg Cd/g food for two weeks. Drobne and Hopkin (1995) observed a reduction of food consumption after 35 days exposure of terrestrial isopods to 2000 μg Zn/g in their food. In another study, Drobne and Hopkin (1994) reported a reduced feeding rate as a result of feeding on Co-dosed food. In this case, 500 μg/g Co in food led to a slight, statistically insignificant effect on the feeding rate, while 2500 μg/g Co in the food significantly reduced the feeding rate after 3 weeks of exposure. Knigge et al. (2000) reported that after 80 days of exposure the applied lead concentrations at a maximum of 7945 mg/kg food dry weight had no significant quantitative effect on food consumption by isopods, although a population pre-exposed in an artillery range showed a tendency towards food uptake higher than that of the control population. Jereb et al. (2003) reported a reduction in the feeding rate after exposure to 300 μg of the radiotracer $^{203}Hg^{2+}$/g leaf for 7 days but no difference in the food consumption was observed in animals that were exposed to 0.3 μg $^{203}Hg^{2+}$/g leaf for 16 days and to 3.0 μg $^{203}Hg^{2+}$/g of leaf for 16 or 35 days.

Feeding rate changes appear to be a suitable measure of effects of ingested chemicals. Whether this is a convenient measure of effects of nanoparticles is needed to be confirmed in future research. Data obtained with nanoparticles suggested that feeding rate changes are neither dose nor time dependent. Feeding rates of exposed animals either increased or decreased when compared to controls. Such result may indicate that: (a) exposure duration was not long enough to provoke effect; (b) exposure concentration was too low to exert effect or (c) nanoparticles have stochastic type of effects which occur by chance and are not time nor dose dependant. To confirm this is a change for future research.

In the study presented here feeding rates were not severely affected even at exposures of up to 28 days, but an effect was seen at shorter exposure duration. Consequently, a concentration of nano-TiO_2 of 2000 μg/g in the food may not be assumed to be a "no observed effect concentration" (NOEC).

In contrast to the not so significant effect on standard toxicity parameters in our study the cell membranes of digestive glands in almost half of exposed animals (42%) fed on 1000 μg/g nano-TiO_2 were destabilized after as little as 3 days of exposure. After 7 days of exposure to food containing 1000 μg/g nano-TiO_2, digestive cell membrane destabilization was detected in 36% of the exposed animals. Animals exposed for longer periods, 14 or 28 days, did not exhibit such intensive membrane damages as was expected, but in animals exposed for 28 days to highest exposure concentration, the digestive gland membrane was severely damaged, a result that was never observed in controls. We conclude that the severe damage of membrane was neither dose- nor exposure duration dependent but occurs sporadically. Here again, we observed differ-

ent type of response to nanoparticles when compared to non-nanoparticulate chemicals. Performed with soluble chemicals, the AO/EB assay reveals a dose response effect (Valant et al. 2009).

A moderate effect was found to be more common in animals exposed to lower concentrations and for shorter times. In the light of currently available knowledge we speculate that in such cases, the cell membrane is destabilized, but the organism has a mechanism to restore its normal activity. The ability of cells to alter their lipid composition and thus their rigidity after exposure to CuO nanoparticles has been demonstrated by Mortimer et al. (2011).

We speculate that in our study, TiO_2 nanoparticles interact first with the cell membrane, and this interaction is diagnosed as cell membrane destabilization. Subsequently, the cells respond to this destabilization of the cell membrane by repairing its stability. This is indicated by the failure to observe intensification of cell membrane destabilization after prolonged exposure durations, such as 28 days. Not with standing this, the cell membrane in some animals was severely affected. Future research is needed to learn if this severe damage could lead to toxic effects or if it can be reversed.

Conclusions

We found nano-TiO_2 to manifest no severe toxicity after 3, 7, 14 or 28 days of dietary exposure to 1000 μg/g or 2000 μg/g of nano-TiO_2 when measured by conventional toxicity measures such as feeding rate, weight change, and mortality.

Severe cell membrane destabilization was sporadic, and was independent of dose and duration of exposure.

The highest tested concentration with 28 days of exposure is not the NOEC because the membrane destabilization effects were observed at shorter duration periods.

The toxic effect of nanoparticles has to be interpreted differently from that of soluble chemicals. It appears more a stochastic-like effect which is not dose responsive.

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Soil ecotoxicology: state of the art and future directions

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Abstract

Developments in soil ecotoxicology started with observations on pesticide effects on soil invertebrates in the 1960s. To support the risk assessment of chemicals, in the 1980s and 1990s development of toxicity tests was the main issue, including single species tests and also more realistic test systems like model ecosystems and field tests focusing on structural and functional endpoints. In the mean time, awareness grew about issues like bioavailability and routes of exposure, while biochemical endpoints (biomarkers) were proposed as sensitive and potential early-warning tools. In recent years, interactions between different chemicals (mixture toxicity) and between chemical and other stressors attracted scientific interest. With the development of molecular biology, omics tools are gaining increasing interest, while the ecological relevance of exposure and effects is translating into concepts like (chemical) stress ecology, ecological vulnerability and trait-based approaches. This contribution addresses historical developments and focuses on current issues in soil ecotoxicology. It is concluded that soil ecotoxicological risk assessment would benefit from extending the available battery of toxicity tests by including e.g. isopods, by paying more attention to exposure, bioavailability and toxicokinetics, and by developing more insight into the ecology of soil organisms to support better understanding of exposure and long-term consequences of chemical exposure at the individual, population and community level. Ecotoxicogenomics tools may also be helpful in this, but will require considerable further research before they can be applied in the practice of soil ecotoxicological risk assessment.

Keywords

Toxicity tests, bioavailability, ecological effects, biomarkers, soil organisms, isopods

Introduction

Ecotoxicology studies the effects of chemicals on organisms in the environment, with the final aim to protect the structure and functioning of ecosystems. This aim generally is achieved by assessing effects on single species of selected test organisms and trying to extrapolate the obtained (no) effect concentrations to safe levels for populations and communities. In the ecotoxicological risk assessment of chemicals, such safe levels are then compared with predicted or measured exposure levels to assess the possible risk for exposed ecosystems.

This paper will give an overview of developments in soil ecotoxicology, focusing on soil invertebrates, starting with a historical overview. Based on that, the state-of-the-art of current soil ecotoxicology will be depicted. This is done by first describing the way soil ecotoxicological data are used in the risk assessment of new and existing chemicals or the assessment of the risks of soil contamination. Next, the development of soil toxicity tests is outlined, followed by considerations on the inclusion of bioavailability, and the use of multiple species, model ecosystem and field tests. Then tools of assessing the possible risk of contaminated soils are described. Finally, some thoughts are given on the future of soil ecotoxicology. As this paper was written on the basis of a presentation at an isopod meeting, special attention will be given to the use of isopods in soil ecotoxicological testing.

Historical perspective of soil ecotoxicology

When thinking of ecotoxicological effects, it is often referred to Rachel Carson, publishing her book 'Silent Spring' in 1962. This book was among the first describing the negative side-effects of the increasing use of synthetic pesticides that started from the second World war onwards. The book mainly focused on pesticide effects on birds, especially singing birds that apparently became silent due to the effects of chlorinated pesticides accumulating in the food chain. This book however, was not unique in ringing the alarm bell, although other bells did not sound that loud.

The first soil ecotoxicological papers date back to the 1960s, reporting observations on the negative effects of pesticides on soil invertebrates (e.g., Fox 1964, Edwards 1969). Similar to developments in aquatic ecotoxicology, these observations triggered the performance of toxicity tests with selected organisms to enable prediction of such side-effects in the field. First results of such toxicity tests, using Collembola and earthworms, were published by the end of the 1960s, also on pesticides (e.g., Ghabbour and Imman 1967, Scopes and Liechtenstein 1967). In the mean time, the Organization for Economic Co-operation and Development (OECD) started developing Guidelines for the testing of chemicals, to support the chemical risk assessment and pesticide registration procedures developed in most Western countries. It took another 15 years before the first toxicity test with soil invertebrates was internationally standardized by OECD, using earthworms and only focusing on short-term (acute) responses like sur-

vival (OECD 1984). In the 1980s and 1990s, the development of soil ecotoxicological tests received more attention, e.g. in the SECOFASE project funded by the European Union, that explored the possibilities of developing toxicity tests with different soil invertebrates, including earthworms, enchytraeids, nematodes, Collembola, staphylinid beetles, mites, centipedes, millipedes and isopods (Løkke and Van Gestel 1998). Several of the methods developed in SECOFASE never made it to standardization, but the project laid a basis for testing new species, using sub-lethal endpoints and including interactions between species.

During the last ten years, there has been a renewed attention for effects of *mixtures* of chemicals in soil (Van Gestel et al. 2011), while the interaction of chemicals with *other stress factors*, like temperature and soil moisture content, also came into focus (Holmstrup et al. 2010). In addition, the available test methods outline below are nowadays applied to new and *emerging chemicals*, especially to determine the toxicity of nanoparticles using earthworms (e.g. Shoults-Wilson et al. 2011a,b; Heckmann et al. 2011; Hooper et al. 2011), Collembola (Kool et al. 2011), and isopods (e.g. Jemec et al. 2008; Drobne et al. 2009; Pipan-Tkalec et al. 2010).

Ecotoxicological risk assessment

In ecotoxicological risk assessment, two approaches can be distinguished. One approach aims at predicting possible effects of (new) chemicals in order to regulate their use or prevent their introduction onto the market. This predictive approach (*prognosis*) uses laboratory toxicity tests to obtain toxicity data that are used to derive safe levels of chemicals in the environment. The second approach is assessing the actual ecological risk or damage in case of pollution. This diagnostic approach (*diagnosis*) enables setting priorities for remediation and risk reduction, and may provide triggers for the management of contaminated land.

Prognosis starts from the paradigms also used in human toxicology. It assumes that the risk of a chemical for ecosystems can be estimated from its toxicity to a number of surrogate test or indicator species, exposed in standard laboratory toxicity tests. These tests aim at assessing toxicity, which is expressed in terms of dose-response relationships for effects on selected endpoints like survival, growth and reproduction. Toxicity is quantified by parameters like LC_{10} and LC_{50} (the concentrations killing 10% and 50% of the exposed test organisms, respectively), EC_{10} and EC_{50} (the concentrations causing 10% and 50% reduction, respectively in a measured endpoint, e.g. growth or number of juveniles produced), and NOEC and LOEC (no-observable and lowest-observable effect concentration, respectively). Since there is no 'most sensitive species' a battery of toxicity tests is needed to obtain proper insight into the potential hazard of a chemical for the ecosystem. In prognosis, the outcome of toxicity tests is used to establish thresholds or safe levels of chemicals in soil, which can be compared with measured or predicted exposure data (soil concentrations) to assess the (potential) risk.

A critical part of this procedure is the derivation of safe levels of chemicals on the basis of available toxicity data. When only short-term (acute) toxicity data are available (usually focusing on survival) or data for a limited number of species, somewhat arbitrary application factors are applied to derive safe levels that should protect ecosystems. For example, when only one or two LC_{50} values are available, a factor of 1000 is applied to the lowest LC_{50} value; this factor should be sufficient to extrapolate from acute to chronic effects (factor of 10), from one or few species to many species (factor of 10), and from laboratory to field (factor of 10). When sublethal toxicity data (usually NOEC or EC_{10} values for effects on e.g. reproduction) are available for 3 or more species, application of a factor of 10 to the lowest value is considered sufficiently protective. When many toxicity data are available (preferably ≥ 8) for species representative of different taxonomic groups (see below), a statistical method may be applied. Such a statistical method is used to construct a species-sensitivity distribution (SSD), which assumes a log-normal or log-logistic distribution of the sensitivities of species in an ecosystem. From such an SSD the 95% lower limit is selected as the safe level. At this level, at least 95% of the species in the ecosystem are supposed to be safe (Posthuma et al. 2002).

Diagnosis may use the same tools as applied for prognoses, but it more heavily relies on ecological methods and environmental chemistry. Basically, toxicity tests or bioassays are used as diagnosis tools to assess toxicity of soil samples from a contaminated site. Results of the bioassays, together with those of chemical measurements and ecological field observations, are used to assess the potential risk of soil contamination. The three tools together form the TRIAD approach (Jensen and Mesman 2006).

Toxicity tests

Both prognosis and diagnosis use toxicity tests, and in both cases a battery of tests is recommended. Criteria to select tests for such a battery have been formulated e.g. by Van Gestel et al. (1997). These criteria among others include:

1. Practicability, referring to the feasibility and cost-effectiveness of a test;
2. Acceptability, including aspects like standardization, reproducibility and statistical validity of a test method as well as its broad chemical responsiveness;
3. Ecological meaning, including sensitivity and ecological realism of the test method.

To obtain a balanced battery of tests, in addition the following criteria need to be taken into account (Van Gestel et al. 1997):

1. Representativeness for the ecosystem to protect: this includes e.g. the representation of organisms having different life-histories, representing different functional groups, different taxonomic groups and different routes of exposure;

2. Representativeness of responses, to make sure responses measured really are relevant for the protection of populations and communities;
3. Uniformity, which refers to the possibility to apply all tests in a battery to the same test media.

By the end of the 1990s and early 2000 toxicity tests, using sub-lethal endpoints like reproduction, were standardized for enchytraeids, earthworms and Collembola by both the OECD and the International Standardization Organization (ISO). But also Environment Canada, the United States Environmental Protection Agency (EPA) and ASTM International (formerly known as the American Society for Testing and Materials) have described similar methods. Recently, for the same organisms, avoidance behaviour tests have been described, while for earthworms and enchytraeids a bioaccumulation test is available. Table 1 provides an overview of the toxicity tests with soil invertebrates available at the moment.

The oldest standardized toxicity test guideline with soil invertebrates, OECD 207 (OECD 1984), describes two short-term toxicity tests, one using 14 days exposure in soil and the other one exposing the worms for 2 days to treated filter paper. Both methods use survival as the only endpoint. The test on filter paper is only rarely applied nowadays, as it does not have any relevance for exposure in soil. It may however, be a

Table 1. A selection of available toxicity tests with soil invertebrates.

Test organism	Species	Duration (days)	Endpoint	Guideline	Reference
Earthworms	*Eisenia fetida*/ *Eisenia andrei*	14	Survival	OECD 207 ISO 11268-1	OECD (1984) ISO (1993)
		28 (+28)	Reproduction	ISO 11268-2 OECD 222	ISO (1998) OECD (2004b)
		2	Avoidance	ISO 17512-1	ISO (2008a)
	Field test, different species	Up to 1 year	Species diversity; abundance	ISO 11268-3	ISO (1999b)
Enchytraeids	*Enchytraeus albidus,* other *Enchytraeus* species	21 (+21)	Survival, Reproduction	ISO 16387 OECD 220	ISO (2004) OECD (2004a)
		2	Avoidance	No standard guidelines	Amorim et al. (2008a,b)
Mollusca	*Helix aspersa*	28	Survival, Growth	ISO 15952	ISO (2006)
Mites	*Hypoaspis aculeifer*	14	Survival, Reproduction	OECD 226	OECD (2008)
	Platynothrus peltifer	14 70	Survival Reproduction	No standard guideline	Van Gestel and Doornekamp (1998)
	Oppia nitens	28	Reproduction	No standard guideline	Princz et al. (2010)
		2	Avoidance	No standard guideline	Owojori et al. (2011)

Test organism	Species	Duration (days)	Endpoint	Guideline	Reference
Isopods	*Porcellio scaber*	28	Survival, growth	No standard guideline	Hornung et al. (1998a,b)
	Porcellionides pruinosis	14	Survival, reproduction	No standard guidelines	Jänsch et al. (2005)
		2	Avoidance	No standard guidelines	Loureiro et al. (2005)
Collembola	*Folsomia candida* *Folsomia fimetaria*	28	Survival, Reproduction	ISO 11267 OECD 232	ISO (1999a); OECD (2009)
		2	Avoidance	ISO 17512-2	ISO (2011)
Insects	*Oxythyrea funesta*	14	Survival	ISO 20963	ISO (2005)
Carabid beetles	*Pterostichus oblongopunctatus; Poecilus cupreus*	Different durations	Adult or larval survival; adult behaviour, respiration	No standard guidelines	Schrader et al. (1998); Bednarska et al. (2010)

useful exposure method for the rapid screening of chemicals, assessing the uptake and/or biotransformation of chemicals or other types of mechanistic research.

Compared to survival, reproduction is a more relevant endpoint when translating effects to the population level. For that reason, an earthworm toxicity test focusing on reproduction has been developed (ISO 1998, OECD 2004b). Although focus is on reproduction, it is essential in this test to also include weight change of the earthworms, since there is evidence for a trade off between reproduction and growth, which may affect their response to toxicants (Van Gestel et al. 1992, Van Gestel et al. 1995). Like for earthworms, the tests with enchytraeids (ISO 2004, OECD 2004a) also focus on reproduction and survival as the endpoint, but for these organisms no separate short-term (acute) and sub-lethal tests were developed. All the reproduction toxicity tests available with oligochaetes typically have test durations of 21-28 days. In case of the earthworm test with *Eisenia fetida* or *Eisenia andrei* and the enchytraeid test with *Enchytraeus albidus*, this means exposing adult worms for 28 and 21 days, respectively, after which they are collected from the soil; the cocoons are incubated for another 28 or 21 days, respectively to enable determining the number of juveniles produced. Nowadays, *Enchytraeus crypticus* seems to be more commonly used for toxicity testing than *Enchytraeus albidus*. Since that species is smaller, adults are not removed from the soil and reproduction is determined after 28 days of exposure. Considering the shorter life cycle of *Enchytraeus crypticus* and its high reproductive output, limiting test duration to 21 days has recently been advocated (Van Gestel et al. 2011).

A standardized test with snails has been developed by ISO (2006), focusing on survival and growth of juveniles snails (*Helix aspersa aspersa*) after 28 days exposure.

Standardized toxicity tests with soil arthropods include the collembolan species *Folsomia candida* (ISO 1999a) and *Folsomia fimetaria* (OECD 2009), the predatory mite *Hypoaspis* (*Geolaelaps) aculeifer* (OECD 2008), and larvae of the insect *Oxythyrea funesta* (ISO 2005). The tests with the collembolans and the predatory mite typically

focus on survival and reproduction after 28, 21 and 14 days exposure, respectively for the parthenogenetic *Folsomia candida,* the sexually reproducing *Folsomia fimetaria* and the predatory mite *Hypoaspis aculeifer*. The test with insect larvae only focuses on survival.

Tests with carabid beetles have been performed using adult *Pterostichus oblongopunctatus* or larvae of *Poecilus cupreus*, but these tests have not been standardized and use different life stages (larvae, adults), endpoints (survival, mobility, respiration) and test durations (from few weeks to several months) depending on the aims of the study (e.g. Schrader et al. 1998, Bednarska and Laskowski 2008, Bednarska et al. 2010).

Also the toxicity tests with the oribatid mites *Oppia nitens* and *Platynothrus peltifer* described in the literature (Van Gestel and Doornekamp 1998, Princz et al. 2010) are not yet standardized. These tests focus on survival and reproduction.

For assessing chemical toxicity to isopods, also no standard test guidelines are available. Nevertheless, isopods are used as test organisms, using different test durations, different routes of exposure (food, soil) and different endpoints. Drobne and Hopkin (1995) described a test exposing *Porcellio scaber* via food and determining effects of zinc on feeding rates. Hornung et al. (1998a) developed a draft test guideline, allowing for exposure both via food and in soil, also using *Porcellio scaber* as the test species. Several different endpoints have been proposed, but considering the difficulty of culturing *Porcellio scaber* in the laboratory survival, growth and food consumption rate so far have been used most frequently (see e.g. Odendaal and Reinecke 2004, Nolde et al. 2006, Kolar et al. 2010). Another interesting endpoint may be related to the composition of the gut microflora of isopods (Drobne et al. 2002). Nevertheless, isopod (*Porcellio scaber*) reproduction also has been proposed as a test endpoint (Hornung et al. 1998b). Successful reproduction isopod experiments have been performed, e.g. by Van Brummelen et al. (1996a), in a 48-week test with *Oniscus asellus*, applying dietary exposure to Polycyclic Aromatic Hydrocarbons (PAHs), and by Lemos et al. (2010) assessing the reproduction toxicity to *Porcellio scaber* of some chemicals with suspected endocrine-disrupting effects. Other isopod species, like *Porcellionides pruinosis*, are more easily cultured in the laboratory, and therefore may be more suitable for performing reproduction toxicity tests (e.g., Jänsch et al. 2005).

Recently, avoidance response was introduced as an easy, fast and sensitive endpoint. For some chemicals avoidance response may be as sensitive as reproduction, while for others it is at least as sensitive as survival. Great advantage of avoidance tests is that they are fast, with test durations of no more than 2 days. Standard test guidelines for avoidance tests have been developed for earthworms (ISO 2008a) and Collembola (ISO 2011), but similar tests have also been performed with enchytraeids (Amorim et al. 2008a,b, Novais et al. 2010), oribatid mites (Owojori et al. 2011) and isopods (Loureiro et al. 2005, Zidar et al. 2005).

In addition to these tests with soil invertebrates, ISO and OECD have also developed a number of toxicity tests with plants, which are important in soil ecosystems as primary producers. Also, several tests are available focusing on the effects of microbial communities or processes performed by microorganisms, like nitrification.

Considering the fact that for a proper risk assessment a battery of tests is desirable, it is important to consider the currently available test methods. The current set of available tests (Table 1) shows an underrepresentation of arthropods in comparison with their abundance in the field when compared with other species like Oligochaetes. And of the available or suggested tests with arthropods, only the one with Collembola has been standardized. Development and international standardization of more toxicity tests with representative arthropod species therefore is highly needed (see criteria for the selection of test species outlined above). The ecological relevance of isopods, their typical routes of exposure (soil, food) and life history characteristics, the possibility to determine different endpoint, and the fact that they have already been used for testing for more than 30 years, make them highly suitable test organisms. Standardization of toxicity tests with isopods therefore is highly recommended.

Bioavailability

For reasons of standardization and to facilitate comparison of results, all standardized tests use a standard soil type: the so-called OECD artificial soil, first introduced in the earthworm acute toxicity test developed by OECD (1984) This artificial soil is composed by mixing readily available materials like sphagnum peat (10%), kaolin clay (20%) and quartz sand (70%); by adding some $CaCO_3$ pH is adjusted to approx. 6.0. The properties of this soil resemble those of a sandy loam soil. Recently, within OECD the use of 5% peat has been advocated when testing pesticides, in order to increase the 'worst case' realism of the artificial test soil for low organic (agricultural) field soils. Also with the aim to increase realism, the SECOFASE project started using the natural LUFA 2.2 soil (Løkke and Van Gestel 1998). The LUFA soils are commercially available from the Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA) in Speyer, Germany. Since that time, LUFA 2.2 standard soil seems to become more commonly used for toxicity tests with soil invertebrates, also because several test species like the collembolan *Folsomia candida* and the enchytraeid *Enchytraeus crypticus* seem to perform as good in this natural soil as they do in artificial soil.

The notion that soil type was important when determining the toxicity of chemicals went along with the increasing insight into the concept of bioavailability: only a fraction of the total amount of chemical in the soil is available for uptake by organisms and therefore of relevance for risk assessment. This was, for instance, demonstrated by Bradham et al. (2006), exposing the earthworm *Eisenia andrei* for 28 days to different soil types spiked with one concentration (2000 mg/kg) of lead (Pb). While in some soils all worms died, in other soils no mortality was seen and in the remainder of the soils only part of the earthworms died. This finding could be explained from the differences in soil properties, especially pH, clay and organic matter content that affected the availability of lead for the earthworms. The pore-water hypothesis or equilibrium partitioning theory was developed to enable linking toxicity of organic chemicals to the concentration available in the pore water (Van Gestel and Ma 1988, 1990). For metals,

application of this approach turned out to be less straightforward because metal speciation in soils is much more complex (Van Gestel 1997) and many factors may affect metal bioavailability in soils (e.g., Allen 2002, Lanno et al. 2004). It nowadays is realized that bioavailability is not a static but rather a dynamic concept (Luoma and Rainbow 2005, Van Gestel 2008). This was demonstrated by Van Straalen et al. (2005), exposing isopods (*Porcellio scaber*) from a metal-contaminated and a non-polluted site to zinc via the food. Both groups showed the same EC_{50} expressed on the basis of zinc concentrations in the diet, but contrary to the expectation effects could not be explained from zinc concentrations in the animals. So, it seems that flux of zinc through the animals rather than total zinc concentration was determining their sensitivity.

These findings also suggest that when considering bioavailability, not only chemical partitioning of chemicals in the exposure medium (soil, food) and pore-water concentrations have to be considered. The biology of bioavailability also needs attention. One aspect of this is the way organisms deal with chemicals. For metals, internal compartmentalization has been shown to be an important aspect (Rainbow 2002), as it determines what fraction of the metal is present in the body in a metabolically available form. Most soil invertebrates have the capacity to sequester at least part of their metal burden in such a way that it does no longer pose a risk. Isopods use the hepatopancreas for a very efficient storage of excess metals (Hopkin and Martin 1982), while earthworms have their chloragogenous tissue that serves the same purpose (Morgan and Morgan 1998, Morgan et al. 1999). As a consequence, both isopods and earthworms show a huge capacity of storing metals like cadmium, which after uptake are hardly eliminated. Other soil invertebrates, like Collembola and beetles, use the midgut epithelium for metal storage. Upon moulting, also the midgut epithelium is renewed enabling these organisms to excrete excess metal (Hopkin 1989). Internal sequestration determines what fraction of the total metal burden in an organism may contribute to its toxicity or is available for trophic transfer to its predators (Vijver et al. 2004). This was for instance demonstrated by Crommentuijn et al. (1994), who found very high Lethal Body Concentrations for cadmium in isopods compared to other arthropods that could be attributed to the highly efficient storage of the metal in an inert form.

Another way organisms may deal with potentially toxic chemicals is by biotransformation. The process of biotransformation aims at making chemicals more hydrophilic and in this way facilitating their excretion. Isopods and Collembola have been shown to be extremely efficient in biotransforming organic chemicals like Polycyclic Aromatic Hydrocarbons (PAHs), which are excreted by these organisms with half lives of approximately 1 day (Van Brummelen and Van Straalen 1996, Howsam and Van Straalen 2003, Stroomberg et al. 2004). Earthworms seem less efficient in doing so. Possible consequence of this rapid biotransformation is that potentially toxic metabolites may be produced. This has been shown in isopods, with even DNA adducts being formed upon exposure to PAHs (Van Brummelen et al. 1996a). This may also lead to long-term damage and possible effects on subsequent generations as has been shown for phenanthrene in the collembolan *Folsomia candida* (Leon Paumen et al. 2008). As

very little is known about such multi-generation effects, further research on the long-term effects of chemicals is urgently needed.

An important biological aspect that may affect the exposure of soil invertebrates to chemicals is their behaviour. Soil by definition is a heterogeneous environment. As a consequence, also the distribution of chemicals in soil is heterogeneous. Chemicals reaching soil by areal deposition for instance accumulate in the topsoil layer, leading to a depth-related concentration gradient as was shown for PAHs in forest soils (Van Brummelen et al. 1996b). Depending on the habitat and mobility, organisms may be more or less exposed to chemicals present in the topsoil layer. Similarly, the effect of pesticides on earthworms was shown to be highly related to their mobility with epigeic and anecic species being much more vulnerable compared to endogeic species. Especially anecic species, like *Lumbricus terrestris*, which come to the soil surface to forage and mate at night, may experience a very high exposure shortly after pesticide spraying (Edwards and Brown 1982). Also in case of spatially heterogeneous soil pollution, behaviour may affect exposure, as was shown for earthworm exposure to copper in a heterogeneously polluted soil by Marinussen and Van der Zee (1996) In the latter study, knowledge of the uptake and elimination kinetics showed to be very helpful in predicting metal concentrations in the earthworms living in a heterogeneously polluted environment. Also in case of isopods, behaviour is an important factor determining exposure. Unfortunately, no research has been done on the chemical exposure of isopods and consequent effects in relation to their behaviour in the field.

Multiple species, model ecosystem (microcosm) and field tests

All standardized toxicity tests with soil invertebrates focus on assessing the effects of chemicals on single species of organisms. To enable assessment of toxic effects in a more realistic setting, micro- or model ecosystems have been developed, ranging from artificially composed set-ups with a number of selected different species introduced in a well homogenized soil (e.g., Burrows and Edwards 2004) to intact soil columns extracted from the field and incubated in the laboratory (e.g., Knacker et al. 2004). Such model ecosystems or microcosms allow assessing effects at the community level, taking into account the interactions between species. Although basically considered single-species tests, earthworm and isopod tests focusing on decomposition or feeding activity in fact also are multispecies tests as in these tests also the interaction with microorganisms in the gut and in the soil or food are important. In addition, the endpoint (decomposition) has high ecological relevance for assessing potential effects on the functioning of the soil ecosystem (e.g. Hobbelen et al. 2006). The only field test available aims at assessing pesticide effects on earthworms (ISO 1999b), but can be combined with a decomposition or litter bag test (Römbke et al. 2003, OECD 2006, Dinter et al. 2008).

Since the introduction of the term Ecotoxicology, the question for "putting more eco into ecotoxicology" has been raised. Some authors even argued that ecotoxicology

should not be seen as a sub-discipline of toxicology but rather as a case of *stress ecology* (Van Straalen 2003). This notion has triggered the focus on more ecologically relevant test designs, integrated approaches including responses at different levels of biological organization, and taking into account the normal operating range of parameters describing the structure and functioning of soil ecosystems.

Since early 2000, with the notion of stress ecology, more complex issues have been receiving attention, with ecological vulnerability, trait-based analysis and effects on functional endpoints (so-called *ecosystem services*) being key items (e.g., De Lange et al. 2009, Saad et al. 2011). The application of these trait-based approaches in soil ecotoxicology on one hand offers promising perspectives, on the other hand it also demonstrates an enormous lack of knowledge on the traits represented by different species and groups of soil invertebrates.

Diagnosis

Many of the tests initially developed for assessing the toxicity of single chemicals are also used for assessing the toxicity of field samples. In addition to the tests mentioned above, a bioassay using the nematode *Caenorhabditis elegans* has been developed by ISO (2010) Such *bioassays* may be applied together with chemical analysis and field observations. The resulting TRIAD approach is a useful tool for the actual risk assessment of contaminated sites (Jensen and Mesman 2006). ISO (2008b) gives guidance on the choice of different bioassays, depending on the purpose of the risk assessment and taking into account aspects like land use.

Other diagnostic tools include effects at the biochemical level. Such *biomarkers* may act as a sensitive, early warning indicator of possible effects at higher levels of biological organization (Spurgeon et al. 2005), and also may provide information on the mode of action of a chemical (Kammenga et al. 2000). Biomarkers may be applied both to organisms captured from the field and to test organisms exposed to field samples under controlled laboratory conditions (see e.g. Van Gestel et al. 2009). Isopods may be used for such biomarker studies (Köhler et al. 1999, Stroomberg et al. 1999, Stanek et al. 2006, Drobne et al. 2008, Lemos et al. 2009), while also their potential of accumulating metals has been proposed as a suitable monitoring tool ('woodlouse watch' scheme) especially in metal-contaminated areas (Hopkin et al. 1993).

Spurgeon et al. (2005), comparing different biochemical endpoints, demonstrated that responses at the gene level were most sensitive. This notion also plays a role in the recent developments of genetic tools (genomics, proteomics and transcriptomics etc.), which has resulted in a vast extension of the ecotoxicological tool box. *Ecotoxicogenomics* nowadays is seen as a tool to enable better understanding of molecular mechanisms of action of chemicals (Snape et al. 2004), while it may provide insight into the way soil invertebrates are able to develop resistance to pollution, e.g. metal or pesticide tolerance (Van Straalen et al. 2011). Ecotoxicogenomics may also help unravelling the mechanisms by which (metal-based) nanoparticles affect organisms, as e.g. was

determined for the nematode *Caenorhabditis elegans* (Roh et al. 2009, 2010). Ecotoxicogenomics may also open the way for developing new diagnostic tools for assessing possible effects of soil pollution (Van Straalen and Roelofs 2008, Nota et al. 2010). Some authors have advocated that ecotoxicogenomics may enable bridging the gap between genes and populations (Fedorenkova et al. 2010). It remains however, uncertain whether time is ready for such 'from gene to population extrapolations' (Van Straalen et al. 2010). In a recent review, Van Straalen and Feder (2012) discuss the possible use of environmental genomics in the ecotoxicological risk assessment of chemicals. Community and population genomics may provide insight into the species composition at different sites and the possible relationship with pollution. Genome scans may also provide information on genetic changes in specific species that have been exposed to contaminated soils over many generations. Gene expression profiling may provide information on toxicant-induced changes in gene expression. The meaning of these changes however, remains unclear as the linkage between gene expression (transcriptomics) and the functioning of the genes (proteomics) often is not straightforward. At the moment, gene expression analysis is applied to only few species for which the genome has more or less completely been described, like the nematode *Caenorhabditis elegans*, the springtail *Folsomia candida* and the earthworms *Lumbricus rubellus* and *Eisenia fetida*, thus limiting wider application. Information on background gene expression is lacking, hampering a proper interpretation of responses under stressed condition. Van Straalen and Feder (2012) therefore conclude that more research is needed before genomics tools can make a sound contribution to the risk assessment of chemicals.

Outlook

Final aim of (soil) ecotoxicology is the understanding of the long-term effects of chemicals on ecosystems. As such, focus on long-term sub-lethal effects is essential, but it also requires detailed understanding of the processes of exposure, uptake, internal processing (metabolism, sequestration) and intoxication in individual organisms as well as the translation of effects to higher levels of biological organization. From the overview presented in this paper, it may have become clear that soil ecotoxicology has shown a tremendous development in the past 40 years. From the initial realization that chemicals may affect soil organisms, through the development of standardized toxicity tests and the use of soil chemistry to develop the concept of bioavailability, soil ecotoxicology has grown to a mature field of science. The incorporation of biochemical and omics tools on one hand and the link with ecology on the other hand, does guarantee that soil ecotoxicology remains an important player in the field of stress ecology. In spite of the promising developments outlined above, the following aspects need further attention in the near future:

Toxicity tests

Although several toxicity tests are available for soil organisms (Table 1), it is obvious that the current battery is not complete and also not well balanced. As mentioned above, it seems there is an under-representation of arthropods. Isopod toxicity testing seems most advanced, while these organisms also represent an ecologically important and relevant group of soil arthropods. In addition, they offer the possibility of exposure via soil and food, while effects may be determined at different levels including biochemical and genomics, individual (growth, behaviour) as well as ecological (feeding activity). It therefore is recommended to put more effort on standardizing isopod toxicity tests for sublethal endpoints. Finally, it has to be noted that the currently available toxicity tests may need adjustment to make them applicable for determining the toxicity of new and emerging chemicals, like nanoparticles.

Bioavailability

For better enabling extrapolation from laboratory tests to the field and among soil types, it is essential to get better understanding of the routes of uptake of chemicals in organisms. This not only requires attention for the chemical aspects, but also needs a greater emphasis of the 'biological' aspects of bioavailability. This may also require paying closer attention to the way organisms are exposed in the field, and attention for the dynamics of exposure and bioavailability.

Kinetics

For a better understanding of bioavailability but also of the toxicity of single chemicals and mixtures, it is essential to increase our understanding of toxicokinetics and toxicodynamics. Such understanding will also enhance the possibilities to extrapolate effects in time and to higher levels of biological organization, like the population level. Kinetics also is of great importance when considering the toxicity of new chemicals, like nanoparticles, that may show changing properties with time as a consequence of aggregation, agglomeration and dissolution processes. Finally, kinetics should not only address whole organisms but should also include the way organisms deal with chemicals internally (biotransformation, sequestration, internal distribution and translocation).

Ecology

For better understanding exposure in the field and predicting ecosystem effects, our knowledge on the ecology of soil invertebrates needs much better development. Such knowledge also is crucial for the description of the normal operating range of structural

and functional endpoints and for the application of trait-based approaches to understand and predict effects of chemicals on soil invertebrate communities and ecosystem services provided by these communities.

Ecotoxicogenomics

For the application of genomics tools in the diagnosis of soil pollution it is essential to better understand the link between gene expression level responses and ecologically relevant endpoints. A better understanding of gene expression responses may also help unraveling the mechanisms of action of chemicals, single and in mixtures, and as such be helpful in predicting toxicity. In the long run, a better understanding of responses at the genomics level may even provide tools for cross-species extrapolation and the development of completely new models for mixture toxicity, especially when combined with toxicokinetics and toxicodynamics data. Genomics tools may also help unraveling the causes of long-term effects of chemicals, e.g. multi-generation effects as a consequence of accumulation of damage in earlier generations. But all these applications will require an enormous amount of information on the meaning of gene expression profiles in relation to background conditions, in relation to chemical exposure both outside and inside the body and related to ecologically relevant endpoints like growth and reproduction.

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