

Facets of Terrestrial Isopod Biology

edited by

Pallietter De Smedt, Stefano Taiti,
Spyros Sfenthourakis, Ivanklin Soares Campos-Filho



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FACETS OF TERRESTRIAL ISOPOD BIOLOGY

edited by Pallieter De Smedt, Stefano Taiti, Spyros Sfenthourakis, Ivanklin Soares Campos-Filho

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Preface – 11th ISTIB: Facets of terrestrial isopod biology

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The 11th International Symposium on Terrestrial Isopod Biology (ISTIB) was intended to take place in 2020 but a world health crisis decided differently. Towards the end of 2019, cases of pneumonia caused by a new coronavirus (SARS-CoV-2) were reported from Wuhan, China. The virus spread quickly and was declared a pandemic in March 2020. At that time, nobody could foresee the severe consequences this pandemic caused in our daily lives. Many countries went into different forms of lockdown and international travel became impossible. Therefore, it was justly decided to postpone the 11th ISTIB to 2021. However, countries around the world went from a first wave, to a second and a third one, and it became clear that an in-person ISTIB would not be possible in 2021 either. The last ISTIB (10th) was held in 2017 in Budapest, Hungary (Hornung et al. 2018), thus repeated postponement of the next meeting meant a gap of at least five years for the terrestrial isopod community to meet again in person. As the world was still suffering from subsequent waves of SARS-COV-2 virus, the terrestrial isopod community decided to meet virtually in 2021 to share their current research and stimulate new collaborations.

Spinicornis, the Belgian Terrestrial Isopod Group, took on the job of hosting this online edition at Ghent University, Ghent, Belgium. *Spinicornis* was established in 2014 with the mission of continuously collecting distribution data of terrestrial isopods from Belgium (<https://www.spinicornis.be>). After five years, they made inventories in every 10×10 km² of the Belgian territory, catching up with neighbouring countries on the knowledge of terrestrial isopod distribution. The data were recently published in an ecological atlas on the terrestrial isopods of Belgium (De Smedt et al. 2020) and in this special issue. Having reached their main goal, this terrestrial isopod group could put all their energy in the organisation of the first online edition of ISTIB. The editors of this special issue thank the members of *Spinicornis*, and in particular Stijn Segers, Gert Arijs, and Pepijn Boeraeve, for their instrumental contribution on practical issues of the congress. *Spinicornis* co-organised the symposium with the Forest & Nature Lab (ForNaLab), Department of Environment of the Faculty of Bioscience Engineering from Ghent University (<https://www.ugent.be/bw/environment/en/research/fornalab>). ForNaLab studies interactions of ecological processes in terrestrial ecosystems focusing on forests and grasslands in temperate regions. The research is tightly linked to forest and nature management and policy. During the past decade, the lab increasingly incorporated invertebrates, including terrestrial isopods, in their research as important components of ecosystem functioning.

The virtual meeting event was a success clearly demonstrating the need of the terrestrial isopod community to stay in contact and seek new collaboration within and across countries. A total of 117 participants from 25 countries worldwide attended the symposium (Fig. 1). The participants listened to 29 oral presentations, including six invited keynote talks by leading experts on different aspects of terrestrial isopod biology. Additionally, 23 posters were presented and discussed in virtual poster rooms. The oral

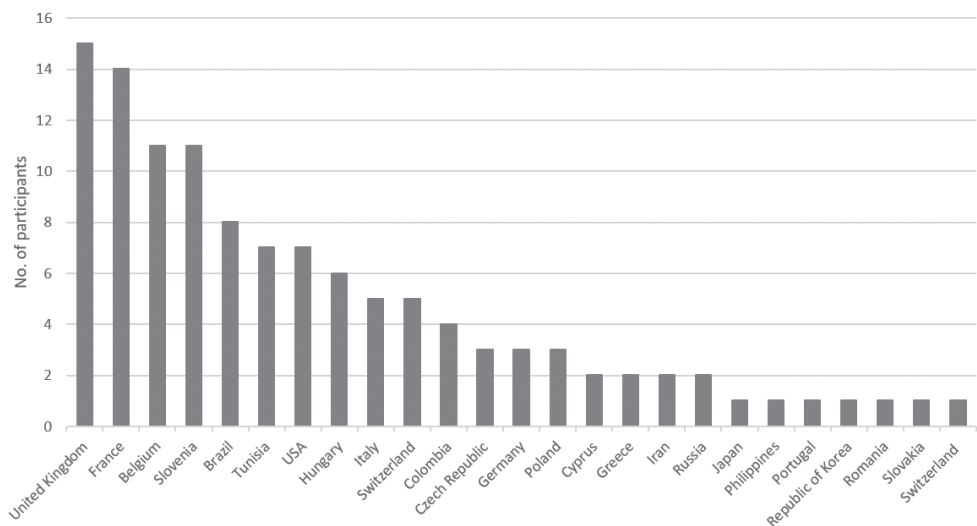


Figure 1. Number of participants per country.

and poster presentations were placed in one of the following six sessions: 1) Taxonomy, phylogeny, and faunistics; 2) Distribution and biogeography; 3) Anatomy and physiology; 4) Host-microbial interactions; 5) Behaviour; 6) Ecology. Although this was a virtual ISTIB, a social event was organized, where participants could meet in rooms and chat in smaller groups while enjoying their favourite drink from their home or work space. This virtual event was preceded by a photograph contest in which all participants could vote (Fig. 2).

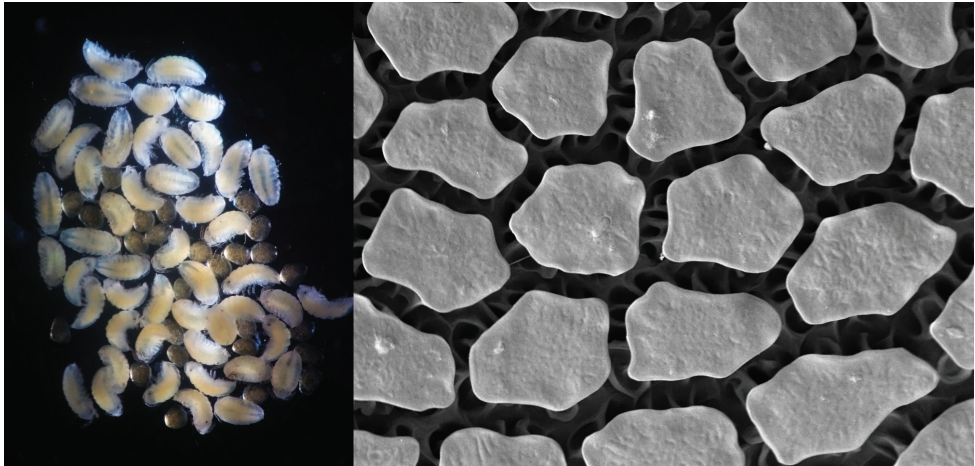


Figure 2. Two of the award-winning photographs of the ISTIB photo competition across the jury and public voting **A** offspring of *Armadillidium vulgare* (Latreille, 1804), winner in the category “Terrestrial isopods”; photograph by Cybèle Prigot **B** the perispiracular cuticle in *Porcellio laevis* Latreille, 1804, winner in the category “Lab research”; photograph by Miloš Vittori.

This special issue “Facets of Terrestrial Isopod Biology” is a collection of twelve papers presented at the 11th ISTIB symposium covering a wide range of topics on recent terrestrial isopod research. The first research paper is a bibliometric analysis of the terrestrial isopod research from the past 70 years which shows how the bulk of terrestrial isopod research is performed and published in developed countries and concentrated in few research labs. Moreover, many of the published research papers are not indexed in “Web of Science”. This review also provides evidence that conference proceedings, such as this issue, are significant contributions to the field. The second research paper describes two species of terrestrial isopods new to science, from Brazilian caves, contributing to the knowledge of cavernicolous isopods from the Neotropical region. A presentation of the database of *Spinicornis* covers the detailed distribution of terrestrial isopods in the Belgian territory. There are three papers on terrestrial isopod behaviour: one studying reactions of terrestrial isopods to high temperature and substrate vibrations, one that studies their behaviour towards toxicity tests, and a third one is a review of the different strategies evolved in terrestrial isopods to avoid predation.

An interesting and rarely studied topic is covered in a paper discussing consequences of immune priming on life history traits of *Armadillidium vulgare* (Latreille, 1804) in the framework of host-microbial interactions. There are two morphological papers, one on the remodelling of septate junctions in the epidermis of *Porcellio scaber* Latreille, 1804 during development, and one on the first observation of intersexuality in the same species. The issue closes with two ecological papers based on nocturnal fieldwork. One paper studies the distribution of terrestrial isopods on a wall in the Czech Republic and the other studies the daily and seasonal activity patterns of the poorly studied *Porcellio albinus* Budde-Lund, 1885 in an arid region in Tunisia. The diversity of these different papers nicely covers different facets of terrestrial isopod biology in a constantly diversifying field (Schmalfuss 2018). This special issue pays tribute to Dr Jonathan C. Wright, a dedicated scientist and terrestrial isopod enthusiast who passed away too soon. The terrestrial isopod community is grateful for his contribution to understanding the relationships between functions of terrestrial isopods and their environment.

We acknowledge that the online format has a different flow and feeling than the in-person meetings we are all accustomed to. At the same time, it provided both the organizers and participants novel symposium experiences that will probably become more common in the future. The virtual meeting also gave the opportunity for researchers from remote countries and/or with limited resources to attend. Until now, all ten previous ISTIB editions were held in or nearby Europe. We keep our hopes high to meet in person again or at least in a hybrid format at the 12th ISTIB, to further advance the research on these intriguing invertebrates that we all highly value as important components of the functioning of our natural world.

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In memoriam Jonathan C. Wright (1961–2019)

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The 11th International Symposium on Terrestrial Isopod Biology and this special issue of ZooKeys is dedicated to the memory of our colleague, Dr. Jonathan C. Wright, who passed away on 16 December 2019. Jonathan was a scientist, educator, musician, public servant, husband, and father.

Life and career

Jonathan was born in Hull, England. His keen interest in natural history since early childhood, motivated him to study zoology, eventually earning a Bachelor's Degree at Lady Margaret Hall in Oxford, then a Master's and PhD at University of Oxford in invertebrate zoology. His first job was with the Nature Conservancy, then Bioscan Ltd., after which Jonathan earned several postdoctoral fellowships. He conducted research at the University of Toronto, University of Copenhagen, McMaster University in Ontario, Canada, and the Marine Biological Laboratory in Woods Hole, Massachusetts. He accepted his first faculty position at Northern State University in Aberdeen, South Dakota in 1993. Five years later, he moved to Pomona College, Claremont California, where he remained a faculty member until his death (Fig. 1). He also served as chair of the biology department in the mid-2000s, and later as Associate Dean of the College for Sponsored Research.



Figure 1. Jonathan Wright at Pomona College. Source: <https://pages.pomona.edu/~jcw04747>

Jonathan's enormous enthusiasm for science and the natural world inspired many students to conduct research projects in his lab; over the years he supervised close to 70 undergraduates, many of whom presented their findings in conferences and became co-authors in scientific publications. His passion in the subject matter, his mentorship and genuine interest in his students' accomplishments have not gone unnoticed: Jonathan was two-time recipient of the Wig Distinguished Professor Award for Excellence in Teaching, as well as the Faculty Alumni Service Award.

Jonathan's research focused on ecophysiology of terrestrial isopods, specifically on water balance, nitrogen excretion, osmotic regulation and thermal balance. He conducted laboratory experiments on many species representing a range of thermal and moisture conditions (Fig. 2). These experiments required analysis of body fluids. Earlier, Jonathan and his colleagues developed a method to sample tiny (nL) amounts maxillary urine, pleon fluid, and haemolymph using a custom-made glass micropipette. Other methodological innovations included a blocking approach to locate where (mouth, rectum, pleopods) water loss/uptake occurs under different environmental conditions, and using inulin tracer to quantify the contribution of haemolymph to water loss during dehydration. These experiments required enormous focus, precision and steady hands. Recognising the importance of early developmental stages in the overall fitness of organisms, Jonathan investigated how isopod embryos in the marsupium tolerate osmotic extremes, ammonia, and pH. In the later years, he focused on patterns of nitrogen excretion, calcium accumulation, and ionic composition of juveniles while still in the marsupium.

The physiological laboratory studies were always framed in a broader, ecological/evolutionary context, and often complemented with field studies. Comparison of species from hygric to xeric habitats revealed the relative importance of water and osmoregulatory mechanisms under different thermal and moisture stress. One study on littoral isopods showed that the discontinuous ammonia excretion is constrained by



Figure 2. Jonathan's research involved taking tiny amount of haemolymph from isopods. Photo: Jaysin Brandt, La Verne Magazine.

the tidal cycle in *Ligia occidentalis*, while in *Alloniscus perconvexus* and *Tylos punctatus*, diurnal cycle regulates the same process.

As a keen zoologist, Jonathan valued 'simple' observations as they are often starting points of generating scientific hypotheses. Such publications or short notes do not generate much interest or citations, yet can later become significant pieces in a jigsaw puzzle. I (KS) am especially grateful for a paper, published in the Proceedings of the South Dakota Academy of Science, in which Jonathan describes winter survival of terrestrial isopods in different microhabitats in South Dakota, with its extreme winter climate. This is one of the early studies highlighting the significance of urban environments as a first step of successful colonisation of areas outside the native ranges of cosmopolitan isopods.

Jonathan's research extended to other invertebrate groups, most notably tardigrades (Tardigrada), but he also conducted experiments on tenebrionids (Coleoptera: Tenebrionidae) and millipedes (Diplopoda). In the later years of his career, Jonathan's focus turned to conservation efforts. His commitment to preserve natural areas and biodiversity motivated him to focus on public service and education. He served in the Claremont Hills Conservation Corporation overseeing how the city manages a nearby

wilderness park. He served as a naturalist guide for Pomona College alumni in California, Alaska, and the Galapagos Island. Jonathan and colleagues were awarded two Henry David Thoreau grants to sustain local conservation efforts.

Jonathan is survived by his wife, Joanne Wright and sons Jeremy and Charlie, his mother Marjorie Wright, sister Lysetta Bray, brother David Wright, and their families. Outside teaching and research, Jonathan was an accomplished musician. He sang in the Pomona College Choir, played the violin in the Pomona College Orchestra, but also performed recitals in a string quartet. Many years ago, I (KS) visited Jonathan in Pomona. He showed me the impressive collection of isopod colonies he kept in the lab, and the experimental setup to conduct these painstaking experiments. We had lunch with Joanne and Jeremy, and then they invited me to their home. With violin and sheet music everywhere, it was evident that music was another important part of Jonathan's life.

Jonathan regularly participated in our symposia, presented and published results on his and his students' research. As a manuscript reviewer, he offered detailed, constructive criticism to the authors. He also kindly edited the language, greatly improving the quality of the manuscript. With Jonathan's untimely passing, the small, close-knit community of terrestrial isopod researchers lost a great colleague.

Reminiscences of happy times

We thought that a story or two about my (THC) several research trips with Jonathan might be a nice addition to this obituary. One such trip was to Australia to find a saline lake near Melbourne that Jonathan had read in some Australian Naturalist's Bulletin as a place to find the uniquely adapted isopod *Haloniscus searlei* (Fig. 3). Despite my misgivings and with credit to Jonathan's sure-minded enthusiasm, we were successful, and also later collected *Ligia australiensis* to study their desiccation resistance.

That trip was followed the next year by several days at the Bamfield Marine Sciences Centre on the west coast of Vancouver Island, where we spent a night on a wild beach in a tiny tent. Jonathan stayed ensconced in relative comfort in the tent while I went out every two hours over a full day and night to collect *Ligia pallasii* for haemolymph sampling. In preparation for the stay we ensured that we had all we needed for every contingency. But when day turned to night as we prepared for much-needed sleep, I said to Jonathan: "one problemo, no alarm clock". He replied that it was no problem, he had a built-in alarm.... Sure enough, every two hours through the night on the dot I felt him nudge me and off I went, thinking to myself who is this superpowered man?

Two years later we were in Hawai'i with two species on our minds, *Ligia exotica* which I knew could be collected in the Honolulu harbour area, and *Ligia perkinsii* that had previously been found off the Pali Escarpment road. We went that evening armed with powerful flashlights to find this highly elusive species. There is not much of a story here except that it was unsuccessful. How does one find an animal that likes to inhabit moist moss on trees? Well, you find them by staring ceaselessly at a portion of moss on a tree, until you eventually see a pair of eyes looking out...then, at the smallest



Figure 3. Jonathan (on the right) and Tom Carefoot looking for a *Haloniscus searllii* near Melbourne, Australia. Photo: courtesy of THC

disturbance, they leap out and, if you are lucky, can be caught in a net. We had more success the next day in finding extra-large *Ligia exotica* on the harbour's surrounding high concrete walls. Jonathan took charge, with him standing at the top and reaching down 2–3m or so with a stick to dislodge them so that they fell into the water, at which time I would fetch them in a leaky old rowboat that we had scavenged. It worked like a dream until we heard a gruff, authoritarian voice yelling, “hey, you fellows, what are you doing here, you’re not allowed in the harbour, and unauthorised use of harbour vessels is not permitted!” Without missing a beat Jonathan took charge, saying something like: “it’s all right sir, we’re from the University following up on a report of an infestation of Japanese *funemushi* in Honolulu Harbour” (I taught him that word courtesy of my university Japanese 101 class). The man was old enough at least to know about the Japanese bombing, and was sympathetic, and all that Jonathan said was true: we *were* from the university and we *were* following up on a report. The fact that the report was from me made it no less true. The next day we found ourselves in a marshy area just south of Pearl Harbor, armed with Jonathan’s haemolymph-sampling equipment: a pin, capillary tubes, a clay-like plugging substance, and a piece of dry ice. We had little else: no food, no water, no flashlight, no mosquito spray, no tent, and nothing to sleep on. We had not actually planned on staying overnight, just following up advice that we should find *Ligia hawaiiensis* there. Well, they were there, in abundance. So, what to do? Jonathan, our man of action, asked for the car keys and disappeared. Within moments it seemed, he was back with an armload of takeout food, flashlights, a collecting bucket, and small net, and believe it or not, a 5 × 8 foot-thick ‘foamy’ that he found at the nearby garbage dump to sleep on. Superman yes, but also Wonderman, all in one. No alarm clock, but then we didn’t need one, did we?! No mosquito spray

either, but it turned out there was none in this marshy, wet, tropical swamp...for all I know arranged by Jonathan! It all went well and, at the end, we had a heart-warming mano-a-mano hug in triumph. Only later did we learn that we should have been very careful, as the area was rife with water-borne parasites. No problem!

From these and other experiences, I found Jonathan to be one of the most knowledgeable biologists I have ever had the pleasure to know, an astute “take-charge” kind of guy, and one of the best research colleagues that I have ever met. In the years following we corresponded regularly and even cooperated on a last publication for me. I miss him greatly.

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A bibliometric analysis of research on terrestrial isopods

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Abstract

Terrestrial isopods (Oniscidea) are crustaceans that thrive in terrestrial environments. This study provides an overview of the major topics in terrestrial isopod research during the last 70 years in order to provide an example of publication practices in invertebrate zoology and to examine how basic research in this area is transferred to its applications. Co-citation analysis and bibliographic coupling based on citation data from the Web of Science Core Collection was used. Findings show that while research on terrestrial isopods expanded in applicative research prioritised by research policies, basic research continues to flourish. The most productive countries in the field include the major developed economies and several smaller nations. In the smaller countries, as well as in France and Italy, the bulk of woodlouse research is performed at a few institutions with traditions in this field. Some of the most influential works have been published in periodicals or monographs that are not indexed in Web of Science or Scopus and lack impact factors. Conference proceedings represent some of the most influential publications in the field. Our findings indicate that smaller and developing economies make significant contributions in invertebrate zoology if their research organisations can achieve continuity of research on a topic. Another conclusion is that journal metrics may be a misleading descriptor of the impact of studies and researchers in this field. Ultimately, these results identify several examples of how basic research in invertebrate zoology leads to applications with considerable socio-economic impact.

Keywords

Citation analysis, crustacean, invertebrate zoology, Oniscidea, scientometrics

Introduction

Invertebrate zoology, as a scientific field, studies the greater part of all living animals, as invertebrates represent the majority of animal biodiversity. Basic research in this field forms the foundation for applicative studies that may extend to other scientific fields and is essential to conservation efforts. Nevertheless, basic research of invertebrates other than model organisms receives relatively little funding and little attention in educational curricula (Leather 2009; Martin 2011). This puts invertebrate zoology in a paradoxical position: although we might expect it to be an extremely important field in the broader scope of the life sciences, it is perceived by the public as almost completely irrelevant. For this reason, it is important to understand how knowledge is shared in the community of researchers in this field and how it is ultimately transferred to its applications.

Terrestrial isopods or woodlice (Oniscidea) are a group of crustaceans that has successfully adapted to the terrestrial environment (Fig. 1). The most recent published account of their diversity lists more than 3700 recognised species, a number that has certainly grown since, making woodlice the most diverse group of isopod crustaceans (Sfenthourakis and Taiti 2015). As litter decomposers, these animals have a profound ecological impact (David and Handa 2010). Woodlice display various degrees of terrestrialisation, making them particularly interesting to the study of evolutionary transition to land (Hornung 2011). In addition to the studies of their diversity and ecology, research interest in terrestrial isopods is very broad, ranging from biogeography to ethology and biochemistry (Schmalfuss 2018).

A comprehensive list of literature on terrestrial isopods published prior to 2000 was assembled by Schmalfuss and Wolf-Schwenninger (2002). The list covers scientific publications dealing specifically with the biology of terrestrial isopods. Schmalfuss (2018) also provided a historic overview of prominent researchers working on terrestrial isopods, from Aristotle to currently active research groups. His survey focused predominantly on research in the field of isopod systematics and the lives and work of some of the giants of woodlouse systematics in the 20th century. It also presented the work of prominent researchers working on other aspects of terrestrial isopod biology. An account of major topics in terrestrial isopod biology was also given in the most recent review of the state of knowledge on these crustaceans, published by Hornung (2011).

In the present contribution, we use bibliometric methods to obtain an overview of the main topics and trends in research dealing with terrestrial isopods, including fields of research that use woodlice as experimental organisms and do not necessarily focus on the biology of this taxonomic group as such. Our aim is to use terrestrial isopod research as a case study of how invertebrate zoology functions in the modern scientific environment and to outline how basic research on invertebrates is linked to its applications. We quantitatively describe the development of this field during the second half of the 20th century and the first two decades of the new millennium and identify the studies that influenced the development of various research directions in the field, including an account of what types of publications they were published in.

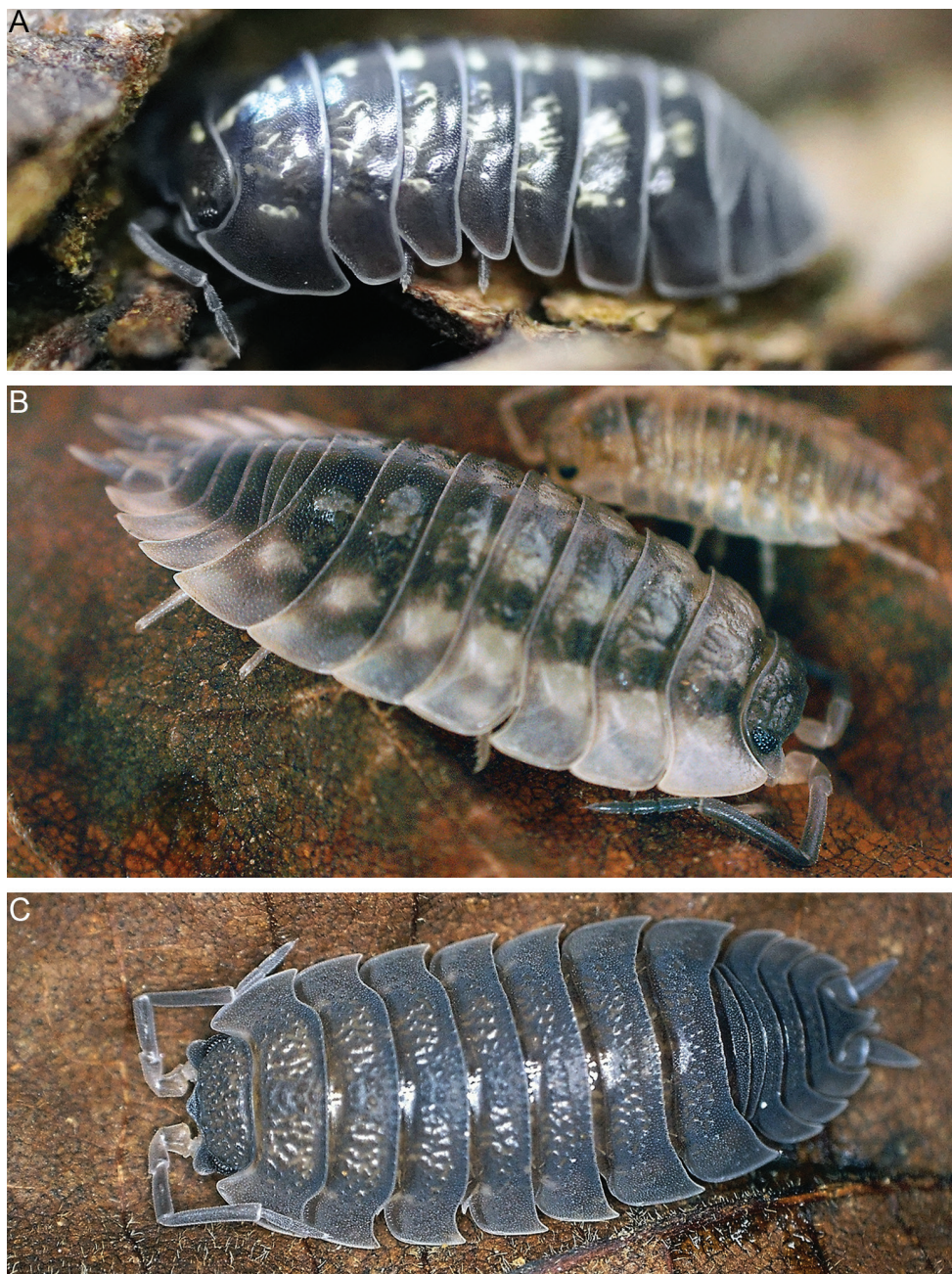


Figure 1. The three most extensively studied woodlouse species (Schmalfuss 2003) **A** *Armadillidium vulgare* **B** *Oniscus asellus* **C** *Porcellio scaber*. Photographs by Ana Sterle (**A**) and Miloš Vittori (**B, C**).

Materials and methods

Bibliometrics are becoming an integral part of research evaluation due to the greater availability of article and citation data, as well as the development of new analysis software (Ellegaard and Wallin 2015). Compared to traditional review techniques, they offer several advantages. Firstly, they are a quantitative way of measuring research impact, meaning that they are objective. Secondly, they are transparent and the results can be replicated using the same method. Thirdly, they are scalable, which means that they can be applied on an individual, institutional, national, or international level. Finally, they allow for the analysis of publication performance, as well as the structure and dynamics of the research field under study.

Our bibliometric analysis relies on citations, which provide an objective measure of a paper's impact in a field of knowledge (Garfield 1979). We use two methods, namely co-citation analysis and bibliographic coupling (Kessler 1963; Small 1978). Each uses citation relationships between publications in its own way, thus complementing each other. Co-citation analysis clusters publications that are often cited together. This property allowed us to identify important publications not included in our database due to being published in non-indexed journals or books as well as works published earlier than the publications in the database. In contrast to co-citation, bibliographic coupling clusters publications with overlapping bibliographies and is thus an adequate technique for the detection of the current state of research, as well as the identification of future trends.

To obtain a relevant dataset of publications on terrestrial isopods, we used the Web of Science Core Collection, which contains publication and citation data. We obtained our dataset by searching for the scientific and trivial names of the taxon Oniscidea, as well as several of the most studied genera of terrestrial isopods that we were able to identify in the World catalogue of terrestrial isopods (Schmalfuss 2003) and the literature overview by Schmalfuss and Wolf-Schwenninger (2002). Our search was limited to the period between 1950 and 2020. While it is possible to search WoS for works published as far back as 1900, few works published before 1950 are indexed in WoS and including this period would not be representative. Earlier relevant publications were therefore identified using co-citation analysis. Upon inspection of the obtained database, we refined the search by excluding keywords most frequently shared by publications that were not relevant to the study.

To obtain our dataset we applied the following search using the appropriate Boolean operators (AND, OR, and NOT):

1. - TS="terrestrial isopod*" OR TS="oniscoid*" OR TS="woodlice" OR TS="oniscid*" OR TS="pill bug" OR TS="sow bug" OR TS="sea slater" OR TS="roly-poly" OR TS="potato bug" OR TS="armadillidium" OR TS="porcellio*" OR TS="philoscia" OR TS="oniscus" OR TS="ligia" OR TS="hemilepistus" OR TS="platyarthrus" OR TS="woodlouse"
2. - TI="crab" OR TI="crayfish" OR TI="decapod" OR TI="daphnia" OR TI="aquatic" OR TI="amphipod*" OR TI="lobster" OR TI="prawn" OR TI="shrimp"

OR TI="gastropod*" OR TI="snail" OR TI="marine isopod" NOT (TI="terrestrial" OR TI="littoral")

3. - TS="mitochondrial" OR TS="androgenic gland" NOT (TS="terrestrial isopod*" OR TS="oniscoid*" OR TS="woodlice" OR TS="oniscid*" OR TS="pill bug" OR TS="sow bug" OR TS="sea slater" OR TS="roly-poly" OR TS="potato bug" OR TS="armadillidium" OR TS="porcellio*" OR TS="philoscia" OR TS="oniscus" OR TS="ligia" OR TS="hemilepistus" OR TS="platyarthrus" OR TS="woodlouse")

4. - TS="random walk" OR TS="navigation" OR TS="path integration"

5. - #1 NOT (#2 OR #3 OR #4)

To visualise bibliometric networks, we used VOSviewer (van Eck and Waltman 2010) and CitNetExplorer (van Eck and Waltman 2014a). VOSviewer can visualise networks of keywords, individual publications, authors, journals, or even countries based on citation, bibliographic coupling, co-citation, and co-authorship relations. To structure a bibliometric map, VOSviewer first uses a co-occurrence matrix to obtain a similarity matrix. Moreover, it constructs a map by locating items close to each other by minimising the weighted sum of the squared Euclidean distances between all pairs of items. Finally, it uses translation, rotation, and reflection to obtain consistent results (Dominko and Verbič 2019). An individual item is assigned to only one cluster and colours are used to distinguish between different clusters. The size of the circle indicates citation strength or occurrence strength in the case of keywords. A more detailed account of the VOSviewer software package can be found in van Eck and Waltman (2010, 2014b). We complemented VOSviewer with CitNetExplorer, which allowed the visualisation of publications on a map where closeness between publications is highlighted on the horizontal axis and the year of publication on the vertical axis. As such, it is an ideal tool for the analysis of the development of a research field. A detailed explanation of CitNetExplorer is available in van Eck and Waltman (2014a).

To interpret the obtained bibliometric networks, items displayed on the maps were looked up in the database, allowing us to identify the topics of the items in question and the journals or proceedings in which they were published.

Results

Publications and citations over time

Our search returned 2946 items related to terrestrial isopods in Web of Science (WoS) and a total of 52631 citations (34880 excluding self-citations). At the beginning of the time period covered by our study in the 1950s, the annual numbers of publications on terrestrial isopods indexed in WoS were fewer than ten and the annual numbers of citations were below 5 (Fig. 2). The publication rate began growing in the 1960s and by 1970 exceeded 20 publications annually. Until the late 1990s, this growth was linear, with annual publication numbers increasing by ca. ten publications every decade.

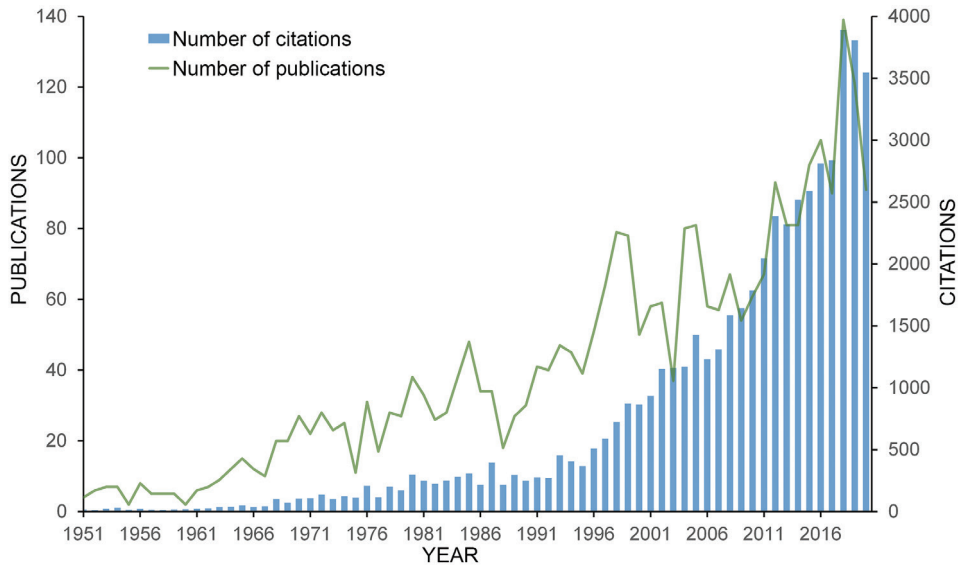


Figure 2. Annual numbers of articles and citations on terrestrial isopods between 1950 and 2020, obtained from Web of Science (WoS).

During the last two decades, however, the rate of publishing increased, doubling from ca. 60 items per year in the early 2000s to roughly 120 per year at end of the 2010s.

The annual numbers of citations of these works followed a similar trend, but citation numbers increased at greater rates than publication numbers. While the annual numbers of citations remained below 30 until the end of the 1960s, the frequency of citation grew steadily to ca. 200 citations per year during the 1970s. At this point, the citation rate reached a plateau and remained unchanged until the mid-1990s. After this point, the annual numbers of citations began to increase rapidly, growing by ca. 1000 annual citations every ten years (Fig. 2).

Numbers of publications according to type

The great majority of indexed publications, more than 88%, are journal articles (Table 1). Reviews account for 3.5%. A surprisingly large percentage of publications is derived from scientific meetings; proceedings papers and meeting abstracts together account for more than 7% of all publications, and proceedings papers are the second most numerous publication type, representing almost 4% of all publications.

Geographical distribution of research on terrestrial isopods

If we take into account publications indexed in WoS, the largest output in the study period comes from the United States with 372 publications. France is not far behind with 357 publications, followed by Germany and the United Kingdom with more

Table 1. Numbers of publications on terrestrial isopods in WoS according to publication type.

Publication type	Number	% of all publications
Article	2597	88.1%
Proceedings paper	111	3.8%
Review	104	3.5%
Meeting abstract	102	3.5%
Note	50	1.7%
Letter	43	1.5%
Other	62	2.1%

than 200 publications each. Other countries with research outputs exceeding 100 publications are Italy, Japan, the Netherlands, Brazil, Slovenia and Canada (Table 2). The majority of the top ten countries in the field are G7 countries, the seven major developed economies (United Nations 2020). This is not surprising as they generally have great research outputs and investments in research and development (National Science Board 2019). The three remaining countries are Brazil, the Netherlands, and Slovenia, a more surprising group. Other prominent countries in the field (Suppl. material 1) include Tunisia, Israel, several members of the European Union, and large developing economies, such as Russia and China. When comparing the productivities of different countries, we should also consider the language bias of international scientific databases. In some countries, a large part of the research output may not be indexed, which may result in an overrepresentation of English-speaking regions (Amano et al. 2016).

Most productive organisations

Almost all among the ten most productive organisations in the field of terrestrial isopod biology (Table 3) are located in the ten most productive countries (Table 2). The exception is the University of Aveiro from Portugal, the eleventh most productive country (Suppl. material 1). The most productive organisation is the University of Poitiers, contributing well over 6% of all publications during the study period. Another French institution among the top 10 is the National Centre for Scientific Research, contributing approximately 1.5% of publications. Italy also has two organisations among the top ten: the National Research Council is the fourth most productive organisation, while the University of Catania took tenth place. The University of Ljubljana (Slovenia) and Vrije Universiteit Amsterdam (the Netherlands) contributed just under 4% of publications each, making them the second and third most productive organisations, respectively.

Considering the contributions of individual organisations, we can deduce that in small countries, such as the Netherlands, Slovenia, Portugal and Tunisia, almost all publications on terrestrial isopods were produced at a single institution. In Italy and France, a few dominant organisations contributed the bulk of the total research output. The same conclusion can be made for most countries outside Europe, such as Brazil, where a handful of institutions contributed the majority of publications (Suppl. material 2). This contrasts with the USA, UK, Japan and Canada, which are among the

Table 2. Numbers of publications on terrestrial isopods by country. The table lists the top ten countries according to the numbers of publications in WoS. These are also the countries with more than 100 publications in the dataset.

Country	Number of publications	% of all publications
United States of America	372	12.63%
France	357	12.12%
Germany	280	9.50%
United Kingdom	270	9.17%
Italy	197	6.69%
Japan	173	5.87%
Netherlands	135	4.58%
Brazil	126	4.28%
Slovenia	122	4.14%
Canada	103	3.50%

Table 3. Top ten organisations that contributed the most publications in WoS in the field of terrestrial isopod biology.

Organisation	Country	Number of publications	% of all publications
University of Poitiers (Université de Poitiers)	France	196	6.6%
University of Ljubljana (Univerza v Ljubljani)	Slovenia	116	3.9%
Free University of Amsterdam (Vrije Universiteit Amsterdam)	The Netherlands	107	3.6%
National Research Council (Consiglio Nazionale delle Ricerche)	Italy	83	2.8%
University of Tunis El Manar (Université de Tunis El Manar)	Tunisia	73	2.5%
University of Aveiro (Universidade de Aveiro)	Portugal	57	1.9%
Ulm University (Universität Ulm)	Germany	53	1.8%
Federal University of Rio Grande do Sul (Universidade Federal do Rio Grande do Sul)	Brazil	53	1.8%
National Centre for Scientific Research (Centre national de la recherche scientifique)	France	40	1.4%
University of Catania (Università degli Studi di Catania)	Italy	36	1.2%

most productive countries, yet individual institutions do not stand out (Table 2 and Suppl. material 2). This suggests that research on terrestrial isopods in these countries is more dispersed among institutions and not linked to the traditions of individual institutions to the same extent as in Mediterranean countries. A similar conclusion can be reached for Germany; while Ulm University stands out in terms of productivity, it nevertheless represents only a fifth of the total German output in this field, with numerous other institutions contributing the remaining publications (Suppl. material 2).

Historic overview

In order to determine the impact of individual publications and which research topics relating to terrestrial isopods were continuously prominent, we conducted a co-

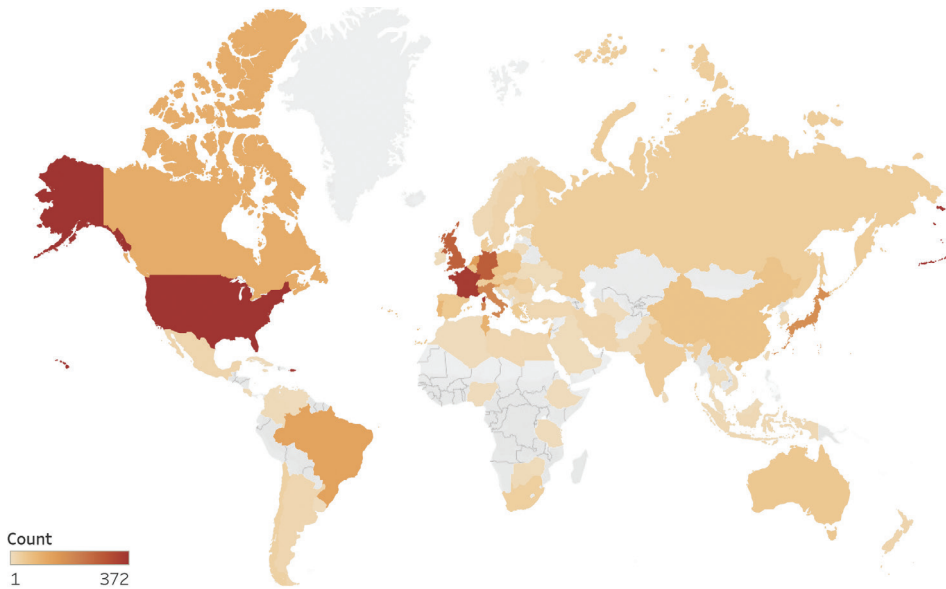


Figure 3. World map of terrestrial isopod research. The colour of each country corresponds to the number of publications on terrestrial isopods in WoS produced by researchers active in that country. Gray areas represent territories without publications in the study dataset.

citation analysis, identifying the publications most often cited by the publications in our database, regardless of whether or not the cited works were included in our WoS-derived dataset. This allowed us to identify relevant works published before 1950 and those published in non-indexed publications. As a result, the period in which these works were published is considerably longer, including most of the 20th century and the first two decades of the 21st century. While many more works on terrestrial isopods were published during this period (see Schmalfuss and Wolf-Schwenninger 2002), our analysis is limited to a few hundred most impactful studies for the sake of clarity.

The visualisation of the co-citation network of the 200 most frequently cited works allows us to discern six major clusters, corresponding to major topics in terrestrial isopod research (Fig. 4). One cluster, shown in purple in Fig. 4, connects works on terrestrial isopod systematics. This cluster is well connected particularly with the cluster of works on woodlouse ecology, physiology and behaviour (shown in red). This broad topic interconnects intensively with another cluster of predominantly physiological studies that focus mostly on digestive physiology and leaf litter decomposition (dark blue). It is not surprising that this digestion research is intimately linked with the green cluster, which connects works on heavy metal accumulation in woodlouse tissues and ecotoxicology. Another cluster, shown in yellow, represents studies dealing with the woodlouse exoskeleton and biomineralisation; this cluster connects with other topics to a lesser extent. Even more distant is the cluster of works dealing with microbe-host interactions, particularly on the feminising bacterium *Wolbachia* (light blue).

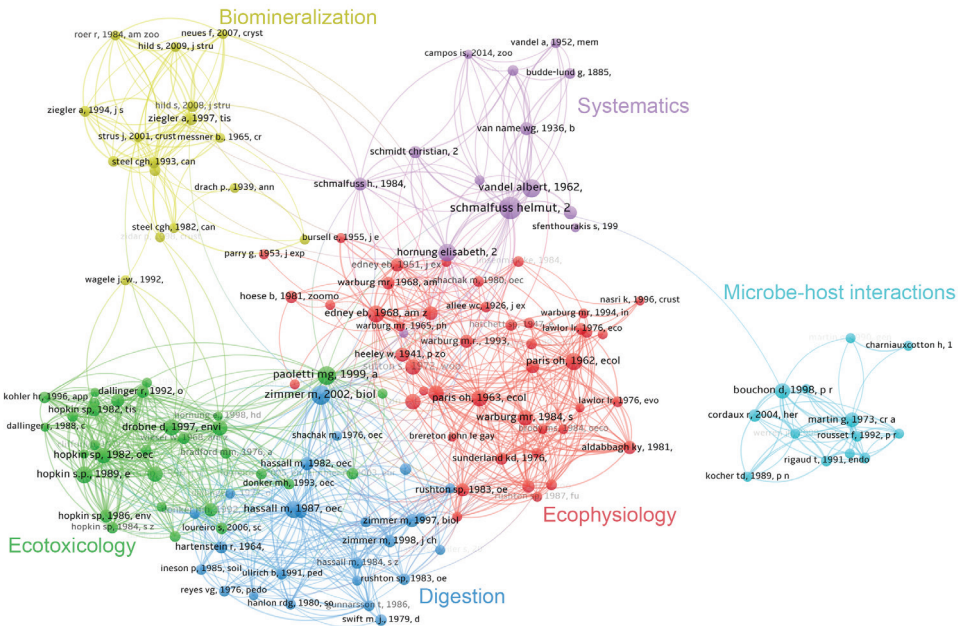


Figure 4. Co-citation network of 200 publications most frequently cited by works in the study dataset. Six major topics of research are discernible (shown in different colours).

By considering a subset of the 100 most influential publications along a timeline, we can identify publications that are predecessors and successors in continuous topics in the field using CitNetExplorer (Fig. 5). On the basis of co-citation, we can identify several clusters of publications that correspond to the major topics of terrestrial isopod research. Before the 1960s, the most impactful publications fall into two clusters (Fig. 5); one (shown in orange) connects works in systematics, while the other (shown in blue) represents works in ecophysiology and encompasses both physiological clusters identified in the broader analysis presented in Fig. 4. In the 1960s, terrestrial isopod research diversified and several additional clusters are identifiable in the co-citation network (Fig. 5). The cluster shown in purple is closely linked to the ecophysiological cluster and represents research on ecotoxicology; another cluster, shown in yellow, deals with biomineralisation, while the green cluster corresponds to works on microbe-host interactions, particularly on *Wolbachia*.

The systematics cluster is relatively scattered and works within it do not interconnect very frequently with each other, suggesting that they are not often cited together. Furthermore, relatively long time intervals elapsed between these works (Fig. 5). An interesting publication is the work of Schmalfuss (1984), which is a part of the systematics cluster but is very distant from other works in this area and instead occupies a central position in relation to most other clusters, establishing numerous co-citation links with them (Fig. 4). This study described the various ecomorphological types of terrestrial isopods and set a framework for numerous comparative studies in other

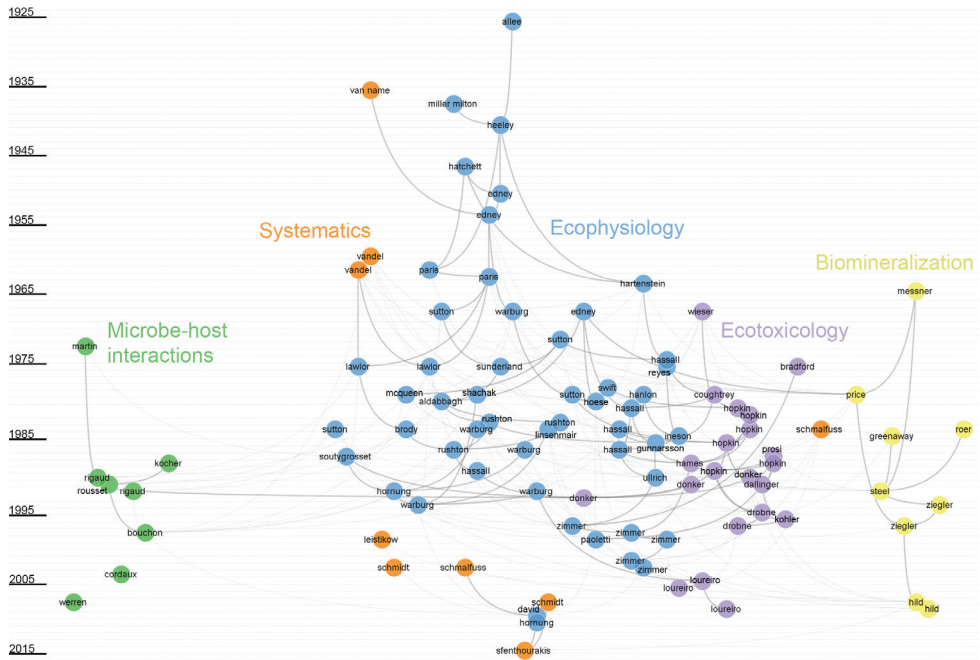


Figure 5. Co-citation network showing the 100 publications that were the most frequently cited by studies in the dataset. The publications are shown along a timeline spanning the period 1925–2015. Names of first authors identify publications.

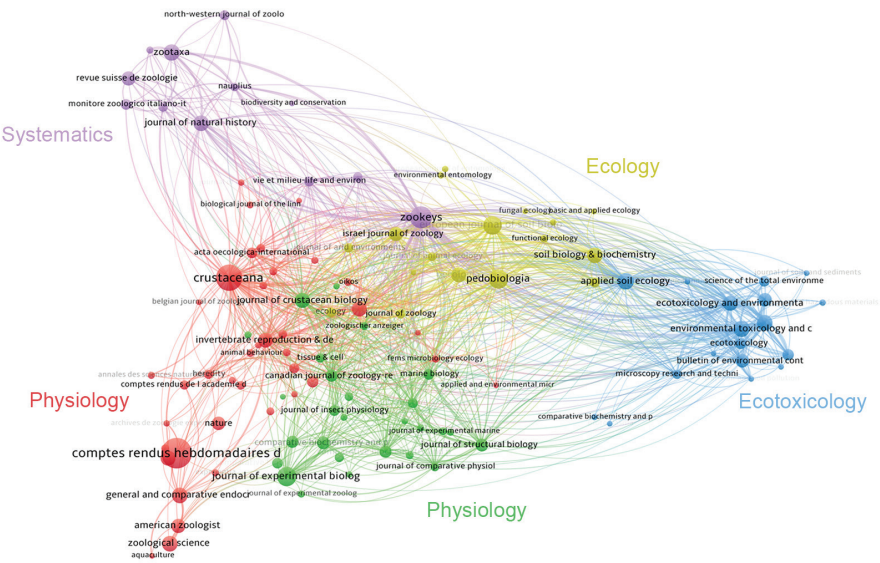
topics. The two volumes of the monograph on terrestrial isopods prepared by Vandel (1960, 1962) hold a similar position. These works presented the morphology of numerous isopod species in great detail. In addition, they also reported on other aspects of isopod biology and thus influenced various research topics in this field.

Numerous links are obvious between systematics and ecophysiology and particularly between this cluster and ecotoxicological publications. By contrast, the clusters dealing with microbe-host interactions and biomineralisation are again more distant from other clusters, which is consistent with the results of the broader co-citation analysis (Fig. 4).

Journals

The topics of journals with the most publications relevant to the field of terrestrial isopod biology fall into five major clusters on the basis of bibliographic coupling (Fig. 6A). The purple cluster encompasses journals that published largely taxonomical and phylogenetic studies in this field. The blue cluster links journals publishing articles on ecotoxicology, while the yellow cluster covers more purely ecological topics. The two remaining clusters, shown in red and green, both cover physiology; while different topics are not very sharply delineated in this case, most of the studies on biomin-

A



B

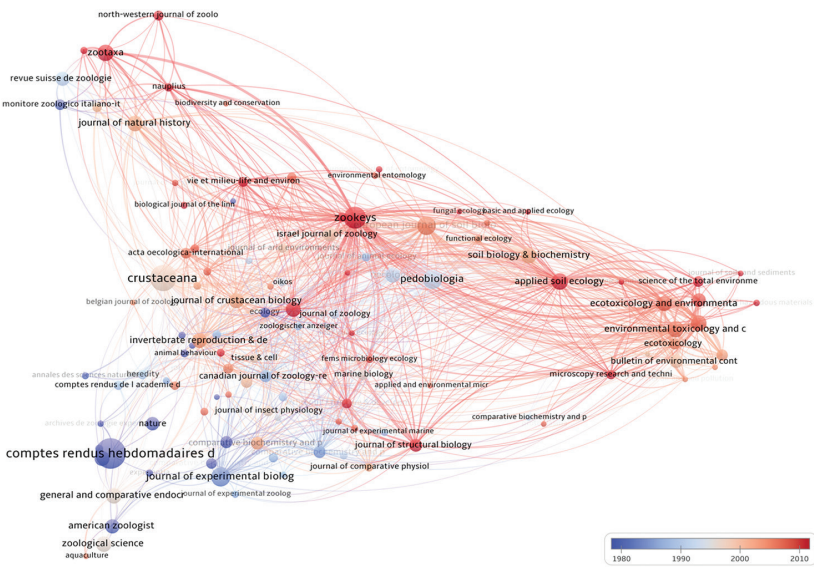


Figure 6. Bibliographic coupling network of journals that have been cited by publications in the database at least 50 times **A** clustering of journals, revealing several major topics that they cover **B** heat-map of the network presented in **A** showing the average year of publication of articles published in these journals and cited in the database.

eralisation and exoskeletal features can be found in journals in the green cluster; the red cluster, on the other hand, encompasses journals that published more works on endocrinology and microbe-host interactions. Understandably, the two physiological clusters are highly interconnected.

Considering the average year of publication of the articles, journals in the ecotoxicological cluster stand out: most cited publications in this area have been published recently (Fig. 6B). The remaining clusters display shifts from certain journals to others during the analysed period. In some of these clusters, such as the ones covering systematics and physiology, a shift is noticeable from regional to international journals. French periodicals were particularly influential in the 20th century; the proceedings of the French Academy of Sciences in Paris ('Comptes rendus hebdomadaires des séances de l'Académie des sciences') and its later offshoot 'Serie D' account for 4% of all publications on terrestrial isopods, more than any other journal (Table 4). While this periodical published the largest body of isopod-related research in the 20th century, its focus later shifted elsewhere.

Apart from ecological and ecotoxicological journals, journals covering systematics, such as 'Zootaxa' and 'ZooKeys', have been very active in the new millennium (Fig. 6 and Table 4). The latter journal publishes thematic issues (Štrus et al. 2012; Taiti et al. 2015; Hornung et al. 2018) dedicated to terrestrial isopod biology in connection with ISTIB (International Symposium on Terrestrial Isopod Biology). This triannual meeting has been bringing terrestrial isopod researchers together regularly since 1983, with some complications only due to the COVID-19 pandemic.

These are the periodicals that published the most articles on the subject of terrestrial isopods, but which periodicals published the most influential studies? When considering the 100 most influential publications (Fig. 3 and Suppl. material 3), the article with the highest citation score was published in 'Stuttgarter Beiträge zur Naturkunde, Serie A', a periodical published by the Stuttgart State Museum of Natural History (Staatliche Museum für Naturkunde Stuttgart). This journal is currently published under the title 'Integrative Systematics'. This periodical is not indexed in WoS and does not have an impact factor. Among other works with the highest citation scores, eleven have been published in the journal 'Oecologia'. These are mostly publications found in the ecophysiological and ecotoxicological clusters identified in the co-citation analysis (Fig. 3). The next most

Table 4. Ten journals that published the most publications on terrestrial isopods during the study period. The entry for the French periodical 'Comptes rendus hebdomadaires des séances de l'Académie des sciences' includes articles published in its 'Serie D'.

Journal title	Published items	% of all items
Comptes rendus hebdomadaires des séances de l'Académie des sciences	120	4.1%
Crustaceana	89	3%
ZooKeys	66	2.2%
Journal of Experimental Biology	49	1.7%
Pedobiologia	48	1.6%
European Journal of Soil Biology	47	1.6%
Applied Soil Ecology	37	1.3%
Environmental Toxicology and Chemistry	37	1.3%
Zootaxa	35	1.2%
Zoological Science	34	1.1%

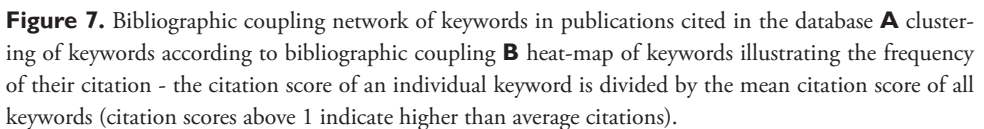
important serial publication, ‘Symposia of the Zoological Society in London’, contributed six articles to the top 100. All six were published in a single publication, the 1984 Proceedings of the First International Symposium on Terrestrial Isopod Biology, making the first ISTIB, which took place in London in 1983, likely the most influential scientific meeting in terrestrial isopod research. These papers set the framework for a large part of studies on isopod biology over the next 40 years. They deal with a variety of topics and are assigned to several clusters in the citation network revealing major topics in woodlouse biology (Fig. 5). Like the most influential journal article, the most influential proceedings papers were thus published in a publication that is not indexed in WoS.

Among other articles in the top 100, three journals published four articles each: ‘Canadian Journal of Zoology’, ‘Environmental Pollution’, and ‘Journal of Animal Ecology’, while two journals contributed four articles each: ‘Soil Biology and Biochemistry’ and ‘American Zoologist’.

Keywords

A network of 100 keywords that appeared in the database most often is presented in Fig. 7, together with a heat-map representation of how frequently articles that included them were cited (Fig. 7B). Keywords fall into five clusters, which correlate to some extent with the study areas identified by co-citation analysis (Figs 4, 5): ecology (red), ecotoxicology (green), systematics with microbe-host interactions (blue), and physiology (yellow). Links are more numerous between keywords related to ecology and ecotoxicology, while keywords relating to systematics, host-microbe interactions and physiology link to other keywords less frequently. Interestingly, keywords linked to microbe-host interactions cluster with systematics keywords, which might be due to the importance of the phylogenetic context to studies on microbe-host interactions. The association between keywords related to microbe-host interactions and the isopod *Armadillidium vulgare* (Fig. 1A) is evident, as is the link between ecotoxicology and physiology with *Oniscus asellus* and *Porcellio scaber* (Fig. 1B, C), likely due to the long-standing tradition of these species as experimental organisms in the respective fields. As can be seen in the citation heat-map, studies on these particular woodlice were cited relatively often. The keywords occurring most frequently refer to the taxon that the work was about, e.g., “terrestrial isopods,” “woodlice,” and “Oniscidea.” These keywords occupy central positions in the network, but as can be deduced from the citation heat-map (Fig 7B), they do not often occur in cited research, possibly due to their generality and the relatively large number of synonymous terms that label the taxon.

Keywords relating to ecotoxicology are consistently frequently cited (Fig. 7B). Among other topics of terrestrial isopod research, keywords linked to microbe-host interactions are highly cited, as well as those dealing with populations, species communities and reproduction. Apart from these, there are some highly cited keywords



relating more generally to invertebrates. These differences in the citation frequencies of keywords lead us to conclude that ecotoxicology, community ecology, and microbe-host interactions are very impactful, likely due to their significance outside the realm of terrestrial isopod research.

Discussion

Our study provides a concise overview of the development of terrestrial isopod research during the last 70 years in terms of how the extensive bibliography on this subject is structured according to publication types and the geographical distribution of publication output.

While the top countries in terms of research output include the leading developed economies with large research expenditures, which can be observed in other scientific fields as well (Holmgren and Schnitzer 2004; Jarić et al. 2012), several smaller and somewhat surprising nations are close to the top, such as Portugal, Slovenia, and Tunisia (Table 2). The high productivity of these smaller countries is linked to a handful of institutions with decades-long traditions in this area of research, as can be deduced if these results are considered alongside data on the most prolific organisations (Table 3) and the historic overview by Schmalfuss (2018). This historic survey is also an excellent source of information for all who are interested in research groups and individuals that made exceptional contributions to the field. Considerable outputs are also produced in BRIC countries: Brazil, Russia, India, and China. France has a prominent position in this field and has been home to some of the most productive researchers and organisations as well as the central journals on the subject in the 20th century. Nevertheless, the majority of the French output is produced by only a few organisations with long traditions in the field, similar to the situation in smaller nations.

The language bias of international scientific databases may distort the representation publications in languages other than English (Amano et al. 2016). This becomes evident in the case of terrestrial isopod biology if we compare the outcome of our analysis of the WoS dataset with the publications published between the years 1850 and 2000 collected by Schmalfuss and Wolf-Schwenninger (2002). Our search in WoS returned fewer than ten publications in individual languages other than English and French (not shown), yet many more such publications can be found in the collection of Schmalfuss and Wolf-Schwenninger (2002) which includes non-indexed publications in a variety of languages. While this does not invalidate our identification of the group of leading nations in the field during the study period, as most of these nations are in a similar position in this respect, the numbers of publications for several countries and institutions are certainly greater than represented in WoS.

By analysing bibliometric networks, we were able to identify several major topics in terrestrial isopod research, the past and current development, and the relationships between these topics. Keyword analysis, co-citation analysis, and bibliographic coupling have identified roughly the same set of general research topics in research related to terrestrial isopods. These are: (1) ecotoxicology, (2) systematics, (3) microbe-host interactions, (4) ecology, with a great focus on population ecology and life histories, and (5) physiology. In the last field, the dominating topics are digestive physiology, ecophysiology (particularly in relation to evolutionary transition to land), and biomineralisation. Many of these topics, such as ecotoxicology, microbe-host interactions, and life-history ecology, have been identified as major topics in this field by authors

who reviewed work on terrestrial isopod biology (Hassall et al. 2005; Hornung 2011; Schmalfuss 2018), whereas some prominent topics, such as ecotoxicology, were not in the scope of those reviews. This is understandable, as these areas of research do not focus on isopod biology but use woodlice as experimental organisms.

Ecotoxicology is an obvious topic on the rise. This is attested by the dominance of this field when it comes to citations and the prominent increase in the number of papers on terrestrial isopods that are published in ecotoxicological journals (Figs 6, 7). The explanation of this success is straightforward, as the field is one of the current priorities of research and development policies, particularly in Europe (European Commission 2020). As we can deduce from the obtained co-citation networks, the initial study influencing this field was the work of Wieser (1968) that deals with the metabolism of metals in the terrestrial environment, largely from the point of view of terrestrials in arthropods. The initial research therefore considered how isopods obtain and conserve metals as micronutrients in the terrestrial environment, in which direct uptake from seawater is impossible. This resulted in the use of woodlice as bioindicators of potentially toxic metals, leading to their extended use in ecotoxicology (Hopkin, 1989). While metal toxicity was the major focus of research at the turn of the millennium, emerging contaminants, such as nanomaterials and microplastics, are now gaining importance (summarised in van Gestel 2012 and van Gestel et al. 2018).

A similar success story is the use of terrestrial isopods in studies on biomineralisation. Here, the initial work in the co-citation network (Fig. 5) is the extensive study on the moulting process in *P. scaber* and *O. asellus* conducted by Messner (1965). Woodlice were quickly recognised as suitable experimental animals for the study of cuticle synthesis and mineralisation due to having several convenient characteristics. Woodlice preparing to moult are identifiable by the presence of sternal calcium carbonate deposits, they moult frequently throughout their lives, and they are relatively easy to maintain and handle in the laboratory. In addition, the need to conserve calcium ions necessary for the mineralisation of their exoskeletons during the process of moulting makes them particularly interesting for the study of mineral dynamics and ion transport (reviewed in Ziegler et al. 2005).

As can be deduced from the importance of the publications and keywords relating to this subject, microbe-host interactions in terrestrial isopods have been a very influential topic as well. A large part of the success of the topic likely results from work on the association of terrestrial isopods with the feminising bacterium *Wolbachia*, which is of great importance to evolutionary biology and the ecology of microbe-host interactions. Although these studies were, to a large extent, conducted on *A. vulgare*, *Wolbachia* is of wider interest as it is capable of infecting numerous arthropods and manipulating their sex. As this is also true for many insects, *Wolbachia* can potentially be used in pest management. This organism is, of course, not the only one studied in woodlice; several commensals and pathogens have been identified in these crustaceans, as well as potential symbionts aiding in the digestion of plant material and other physiological processes (reviewed in Bouchon et al. 2016). Ultimately, research on these associations is still very intensive and facilitated by recent methodological advances in the study of microbiomes (Bredon et al. 2018, 2020).

The examples of research on ecotoxicology, biomineralisation and microbe-host interactions in terrestrial isopods show how basic research on the biology of an invertebrate group later led to the establishment of the animals in question as experimental organisms in more general, even applicative, research topics. Naturally, other topics in woodlouse research had a broader impact on human knowledge as well, as we can expect from any well-conducted scientific inquiry, and these two cases were only discussed at length here as the most obvious examples in the bibliographic networks we obtained.

Despite poor funding opportunities in the field of invertebrate systematics (Martin 2011), there is vibrant evolutionary and taxonomical work on terrestrial isopods being conducted globally, which is reflected in the popularity of systematics-related journals in this field and the universal presence of a systematics cluster in the bibliometric networks obtained in this study. Furthermore, there is no apparent negative trend in the output or the impact of such studies (Figs 5, 6, 7). The relatively scarce knowledge of the diversity of woodlice, particularly in the tropics, the importance of this group from the point of view of animal terrestrialisation (Sfenthourakis and Taiti 2015; Taiti 2017), and perhaps the captivating nature of terrestrial isopods may be factors contributing to the vitality of their systematics.

As demonstrated by the co-citation analysis, the central publications in the field of terrestrial isopod biology during the last 50 years were often conference proceedings and articles published in institutional periodicals, many of which are not indexed in WoS or Scopus and lack impact factors. While the importance of scientific meetings is diminishing and conference proceedings resonate only briefly in most scientific fields (Liséé et al. 2008), symposia on terrestrial isopod biology have a large and lasting impact that is reflected in meetings contributing the most influential publications. At this point, it is too early to make any conclusions about whether or not this trend has continued in the last decade, but proceedings papers are evidently still important to the field, as can be deduced from the intensive bibliographic coupling of the journal that publishes them (Fig. 6). The ultimate importance of proceedings papers and articles in local periodicals shows that as far as terrestrial isopod biology goes, journal metrics do not likely reflect the influence that the publications ultimately have. By extension, the impact factors of journals in which invertebrate zoologists publish their work are poor predictors of the impact these researchers have on their field, a fact we fear is insufficiently appreciated.

Conclusions

The findings of this study point out that publishing in invertebrate zoology follows somewhat specific principles, with great importance of in-person meetings and publications not captured by conventional bibliographic metrics. In addition, major contributions in this field are produced in small and developing economies at institutions that can achieve continuity of research on a topic despite changing research trends. Ultimately, the example of terrestrial isopods shows that basic research on the biology of

a seemingly marginal group of invertebrates can lead to flourishing applicative research on some of today's most pressing issues. This is all the more reason not to neglect such research in the future, as the findings of today can help resolve the issues of tomorrow.

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Supplementary material I

Number publications according to country

Authors: Miloš Vittori, Miha Dominko

Data type: Bibliometric data.

Explanation note: A table listing countries with corresponding numbers of publications on terrestrial isopods in the period between 1950 and 2020.

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Link: <https://doi.org/10.3897/zookeys.1101.81016.suppl1>

Supplementary material 2

Number of publications according to institution

Authors: Miloš Vittori, Miha Dominko

Data type: Bibliometric data.

Explanation note: A table listing institutions with corresponding numbers of publications on terrestrial isopods in the period between 1950 and 2020.

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Supplementary material 3

Most influential publications on terrestrial isopods

Authors: Miloš Vittori, Miha Dominko

Data type: Bibliometric data.

Explanation note: A table listing 100 publications on the subject of terrestrial isopods with the highest citation scores.

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Expanding the knowledge on the diversity of the cavernicolous Styloniscidae Vandel, 1952 (Oniscidea, Synocheta) from Brazil, with descriptions of two new species from the semiarid karst regions

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Abstract

Two new species of *Pectenoniscus* from two caves in karst areas of the Brazilian semiarid region are described. *Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, **sp. nov.** from Gruna do Govi cave, Serra do Ramalho karst area, state of Bahia, and *Pectenoniscus fervens* Campos-Filho, Taiti & Bichuette, **sp. nov.** from Toca Coroa do Frade cave, Barra Bonita karst area, state of Piauí. In addition, specimens of *Cylindroniscus flaviae* from Gruta da Tapagem (= Caverna do Diabo), Açungui karst area were also recorded. An updated diagnosis of *Pectenoniscus* and a distribution map of the species examined herein are given.

Keywords

Açungui geomorphological group, Bambuí geomorphological group, Casa Nova geomorphological group, *Cylindroniscus*, Neotropical, *Pectenoniscus*

Introduction

Terrestrial isopods (Oniscidea) comprise approximately 4,000 species and more than 500 genera distributed in 38 families (Sfenthourakis and Taiti 2015; Dimitriou et al. 2019; Campos-Filho and Taiti 2021). The Oniscidea are one of the most representative taxa in the Brazilian subterranean environments, due to their favourable habitat conditions with high humidity and many different substrates and micro-habitats (Fernandes et al. 2016, 2019). To date, more than 210 species are known from Brazil, of which 70 have been recorded from caves. Among them, 31 species are considered troglobites (obligatory and restricted cave-dwellers) and several are troglophiles (facultative cave-dwellers) or troglonexes (epigean species with individuals using subterranean resources) (Trajano 2012; Trajano and Carvalho 2017; Campos-Filho et al. 2018, 2019, 2020; Cardoso et al. 2020a, b, 2021). However, both troglobitic and troglophile species are not assigned with certainty to these categories due the lack of sampling outside caves. Endogean species exhibiting classical troglomorphic characters, such as lack or reduction of body pigments and eyes, might also occur in the unconsolidated substrate outside caves (Campos-Filho et al. 2014).

The family Styloniscidae comprises 120 species distributed in 18 genera (WoRMS 2021). The family has a worldwide distribution, with species inhabiting many terrestrial environments, including caves (Schmalfuss 2003). Fifty-eight species distributed in 13 genera have been recorded from caves, i.e., *Bamaoniscus* Taiti & Montesanto, 2020 (1 sp.), *Chaimowiczia* Cardoso, Bastos-Pereira, Souza & Ferreira, 2021 (2 spp.), *Clavigeroniscus* Arcangeli, 1930 (2 spp.), *Cordioniscus* Gräve, 1914 (15 spp.), *Cylindroniscus* Arcangeli, 1929 (5 spp.), *Indoniscus* Vandel, 1952 (1 sp.), *Iuiuniscus* Souza, Ferreira & Senna, 2015 (1 sp.), *Pectenoniscus* Andersson, 1960 (7 spp.), *Spelunconiscus* Campos-Filho, Araujo & Taiti, 2014 (1 sp.), *Styloniscus* Dana, 1853 (7 spp.), *Thailandoniscus* Dalens, 1989 (3 spp.), *Trogloniscus* Taiti & Xue, 2012 (5 spp.), and *Xangoniscus* Campos-Filho, Araujo & Taiti, 2014 (8 spp.) (for all recorded species see Dalens 1987; Mulaik 1960; Schultz 1970, 1995; Green 1971; Vandel 1973, 1977, 1981; Ferrara and Taiti 1979; Taiti et al. 1992; Taiti and Howarth 1997; Schmalfuss and Erhard 1998; Andreev and Bozarova 2000; Andreev 2002; Green et al. 2002; Taiti and Xue 2012; Campos-Filho et al. 2014; Souza et al. 2015; Bastos-Pereira et al., 2017; Fernandes et al. 2018; Cardoso et al. 2020a, b, 2021; Taiti and Montesanto 2020).

To date, in Brazil, 26 species of the family distributed in nine genera have been recorded from caves, i.e., *Chaimowiczia* (2 spp.), *Clavigeroniscus* (1 sp.), *Cordioniscus* (1 sp.), *Cylindroniscus* (2 spp.), *Iuiuniscus* (1 sp.), *Pectenoniscus* (8 spp.), *Spelunconiscus* (1 sp.), *Styloniscus* (2 spp.), and *Xangoniscus* (8 spp.) (Campos-Filho et al. 2018, 2019; Cardoso et al. 2020a, b, 2021). It is worth mentioning that the Styloniscidae in Brazil hold the highest diversity of troglobitic isopods comprising 20 out of 31 species (Campos-Filho et al. 2018, 2019, 2020; Cardoso et al. 2020a, b, 2021).

Two new species of *Pectenoniscus* from caves of two distinct Brazilian karst regions are described here. The first species comes from Gruna do Govi, Serra do Ramalho karst region, inserted in the Bambuí geomorphological group, state of Bahia, and the second

from Toca Coroa do Frade, Barra Bonita karst region, Casa Nova geomorphological group, state of Piauí. In addition, specimens of *Cylindroniscus flaviae* Campos-Filho, Araujo & Taiti, 2017 from Gruta da Tapagem (also known as Caverna do Diabo), Açungui geomorphological group, are recorded here. An updated diagnosis of the genus *Pectenoniscus* is given to include the species described by Cardoso et al. (2020b) and the two new species. Ecological and conservation remarks considering IUCN threat categories, are provided.

Materials and methods

Collections and taxonomy

Specimens were collected by hand with the aid of tweezers and brushes and stored in 70% and 100% ethanol. Information about the microhabitat (entrance, twilight or aphotic zones) and environmental variables (temperature and relative air humidity) of the caves was also recorded. Descriptions are based on morphological characters with the use of micro-preparations in Hoyer's medium (Anderson 1954). For each new species, the diagnosis, type material, description, etymology and remarks are given. The *habitus* images were taken with the stereomicroscope model Motic SMZ-168 and the Celestron Microcapture Pro. The photographs were prepared with Adobe Photoshop CC Lite (v. 17.1.1). The appendages were illustrated with the aid of a camera lucida mounted on a CH2 Olympus microscope. The final illustrations were prepared using the software GIMP (v. 2.8) with the method proposed by Montesanto (2015, 2016). A map highlighting the caves where all species occur, as well the hydrological attributes and pressures of economic activities in the region, is presented. The distribution map was constructed with the QGIS software (v. 3.18.1) and the final edition with PowerPoint Microsoft 365 (v. 2108).

The material is deposited in the scientific collection of cave fauna of the Laboratório de Estudos Subterrâneos (**LES**), Universidade Federal de São Carlos, São Carlos, Brazil.

Study areas

Parque Estadual Caverna do Diabo, Açungui geomorphological group

The Açungui geomorphological group comprises the metamorphic limestone and dolomite rocks of ~ 600 million years ago, extending from south of the São Paulo state to north of the Paraná state (Rubbioli et al. 2019). Due to the altitudinal range, this group has one of the largest concentrations of irregular limestone areas in the country, including very ornamented caves crossed by rivers (Rubbioli et al. 2019). The Açungui group is located in the Chacoan subregion, in all provinces of the Parana domain, i.e., Atlantic Forest, *Araucaria* Forest, and Paraná Forest (Morrone 2014). According to Köppen's criteria, it shows a warm temperate climate, fully humid with warm summer (Kottek et al. 2006).

This region includes the Caverna do Diabo State Park (PECD, in Portuguese, Parque Estadual da Caverna do Diabo), state of São Paulo (Fig. 1), which covers the

municipalities of Barra do Turvo, Cajati, Eldorado and Iporanga (Fundação Florestal, 2010). The PECD was created in 2008 and it has more than 40,000 ha, constituting the Jacupiranga mosaic of conservation units (Fundação Florestal 2010). Gruta da Tapagem, also known as Caverna do Diabo (Fig. 2A), is ~ 8 km long and it is considered one of the most important caves of the PECD. The cave is inserted in the Tapagem dolomitic marble, in the André Lopes carbonate belt, and it is a sinkhole of the Ribeirão da Tapagem, a river which develops its subterranean course in ~ 4 km to the resurgence in the Vale do Rio das Ostras, a right-bank tributary of the Ribeira de Iguape (Karmann and Sánchez 1979; Hiruma et al. 2008; Rubbioli et al. 2019). The temperature and relative humidity of the air of the cave ranged from 28.8 °C and 60% RH in the entrance zone to 26.5 °C and 78% RH in the aphotic zone. This cave is notable for its scenic beauty with large halls and speleothems, and a stretch with illumination, stairs, and walkways for touristic activity (Silverio 2014).

Serra do Ramalho karst area, Bambuí geomorphological group

The Bambuí geomorphological group has the largest limestone area (ca. 146,000 km²) and the highest number of caves in Brazil (Rubbioli et al. 2019). The group includes the Serra do Ramalho karst area, located in the southwestern of the state of Bahia and the municipalities of Coribe, Feira da Mata, Carinhanha and Serra do Ramalho (Rubbioli et al. 2019). This area is inserted in the middle of the São Francisco River basin, dominated by a plateau of carbonate rocks with a high number of caves, mostly without legal protection (Auler et al. 2001; Rubbioli et al. 2019). According to Köppen's criteria, the climate is tropical dry, characterised by dry winters and annual precipitation of ~ 640 mm (Bedeck et al. 2018, 2020). The dominant vegetation is “Caatinga”, composed of mesophytic and xeromorphic forests interspersed with “Cerrado” (savannah-like vegetation) (Bichuette and Rizzato 2012).

The Gruta do Góvi (Figs 1, 3) is located in a private property of the municipality of Feira da Mata. The surrounding of the cave harbours native vegetation and pastures, and anthropic impacts like garbage, graffiti on the walls and systems for capturing water from the subterranean drainage (Fig. 3C, D).

Barra Bonita karst region, Casa Nova geomorphological group, state of Piauí

The Barra Bonita karst region is inserted in the Casa Nova geomorphological group, and it is formed by quartzites, mica schists, and limestones of ~ 740 Myr (Rubbioli et al. 2019). The limestone area has a restricted occurrence in southeastern of the Piauí state, surrounded by “Caatinga” as typical vegetation domain, annual temperatures ranging from 25 °C to 31 °C, and 689 mm of average annual precipitation (Nascimento & Mantesso-Neto 2013; Hadler et al. 2018).

The Gruta Toca Coroa do Frade (Figs 1, 2C, D) is located in the municipality of Coronel José Dias, outside of the Parque Nacional da Serra da Capivara. The temperature and relative humidity of the air of the cave ranges from 30.9 °C and 49% RH in the entrance zone to 31 °C and 63% RH in the aphotic zone.

Systematic account

Genus *Cylindroniscus* Arcangeli, 1929

Type species. *Cylindroniscus seurati* Arcangeli, 1929 by monotypy (see Schmidt and Leistikow 2004).

Cylindroniscus flaviae Campos-Filho, Araujo & Taiti, 2017

Figs 1, 2B

Cylindroniscus flaviae Campos-Filho, Araujo & Taiti, in Campos-Filho et al. 2017a: 229, figs 1–5.

Cylindroniscus flaviae; Campos-Filho et al. 2017b: 70; Campos-Filho et al. 2018: 4; Fernandes et al. 2018: 441; Silva et al. 2018: 56.

Material examined. BRAZIL●1♀, Gruta da Tapagem (Caverna do Diabo), Eldorado, Parque Estadual Caverna do Diabo, Açungui geomorphological group, state of São Paulo, 24°38'17.00"S, 48°24'4.00"W, leg. ME Bichuette, T Zepon, JE Gallão, 24.III.2021, LES 27755●1♀, same locality and collectors as for preceding, 24.III.2021, LES 27756●1♂, 1♀, same locality and collectors as for preceding, 24.III.2021, LES 27757●1♀, same locality and collectors as for preceding, 24.III.2021, LES 27758●2♀♀, same locality and collectors as for preceding, 24.III.2021, LES 27759●1♂, same locality and collectors as for preceding, 25.III.2021, LES 27760.

Remarks. *Cylindroniscus flaviae* shows preference for organic matter deposits and highly humid areas in the aphotic zone (Fig. 2B). The organic matter was observed in several conduits of the Gruta da Tapagem, always far from the touristic stretches, and it was composed of particulate vegetal debris or small tree branches. The environmental variables along the cave ranged from 19.9 °C to 20.4 °C and the relative air humidity from 95% to 99.1%. The individuals demonstrated sensitivity to the flash lights of the lanterns, always moving in opposite direction.

Distribution. This species is recorded from several caves in the Açungui geomorphological group (see Campos-Filho et al. 2017).

Genus *Pectenoniscus* Andersson, 1960

Type species. *Pectenoniscus angulatus* Andersson, 1960 by monotypy (see Schmidt and Leistikow 2004).

Diagnosis. After Andersson (1960) and Campos-Filho et al. (2019). Animals of reduced size, ≤ 3.5 mm. Body unpigmented and eyes absent. Body slender with lateral sides almost parallel. Dorsal surface of cephalon and pereon bearing small transverse tubercles, conferring granulated appearance, pleon smooth or slightly tuberculate. Cephalon with 4–6 rows of tubercles, gradually reducing in number from posteri-

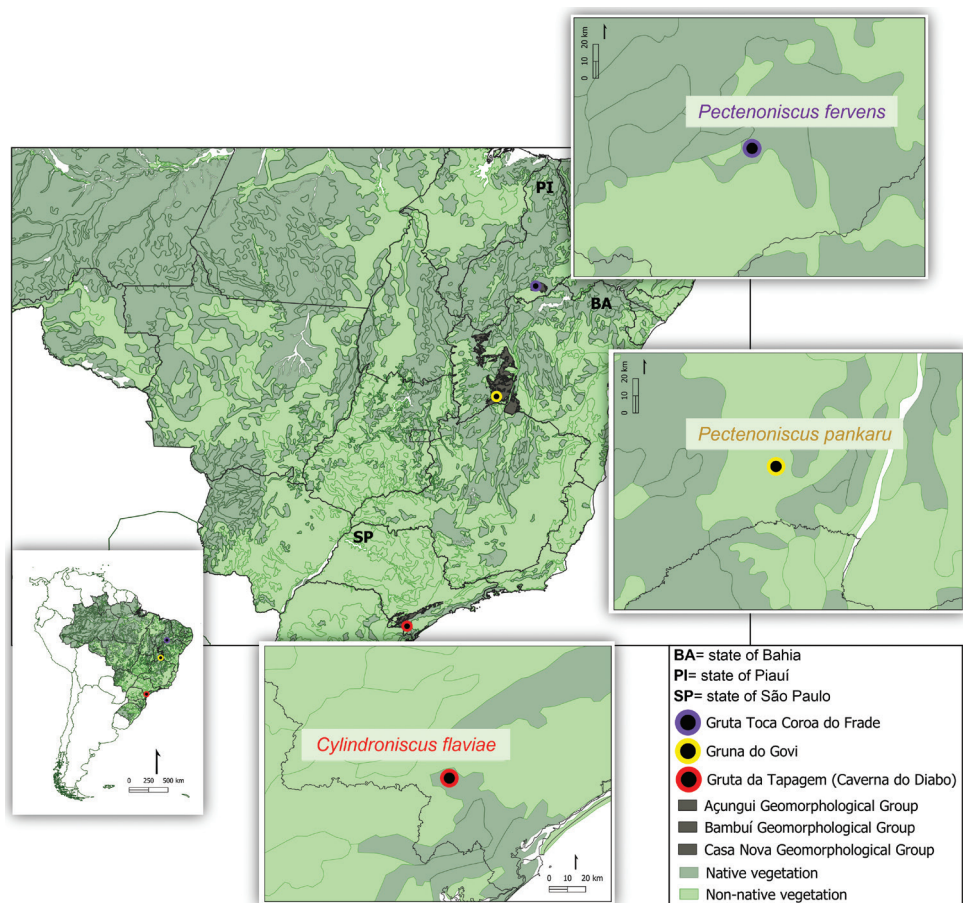


Figure 1. Distribution map of *Cyldroniscus flaviae* Campos-Filho, Araujo & Taiti, 2017, *Pectenonsiscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov., *Pectenonsiscus fervens* Campos-Filho, Taiti & Bichuette, sp. nov.

or to distal portion, pereonite 1 with two or three rows of tubercles, pereonites 2–7 with two rows of tubercles. Dorsal scale-setae triangular. Cephalon with antennary lobes and suprantennal line. Pleonites 3–5 epimera not developed (only developed in *P. angulatus*). Telson triangular with lateral sides concave and rounded apex. Antennula of three articles, distal article with aesthetascs arranged in one longitudinal row. Antennal flagellum of 3–5 articles. Mandibles with strong molar process, left mandible with two penicils, right mandible with one penicil (sometimes one penicil on molar process). Maxillula inner endite with three penicils, proximal one longest; outer endite composed of eight or nine teeth plus slender stalks. Maxilla of two lobes covered with thick and fine setae, inner lobe wider. Maxilliped basis with lateral sides almost parallel, endite rectangular bearing one stout penicil. Uropod protopod subquadrangular, exopod longer than endopod, protopod and exopod sometimes bearing glandular pores,

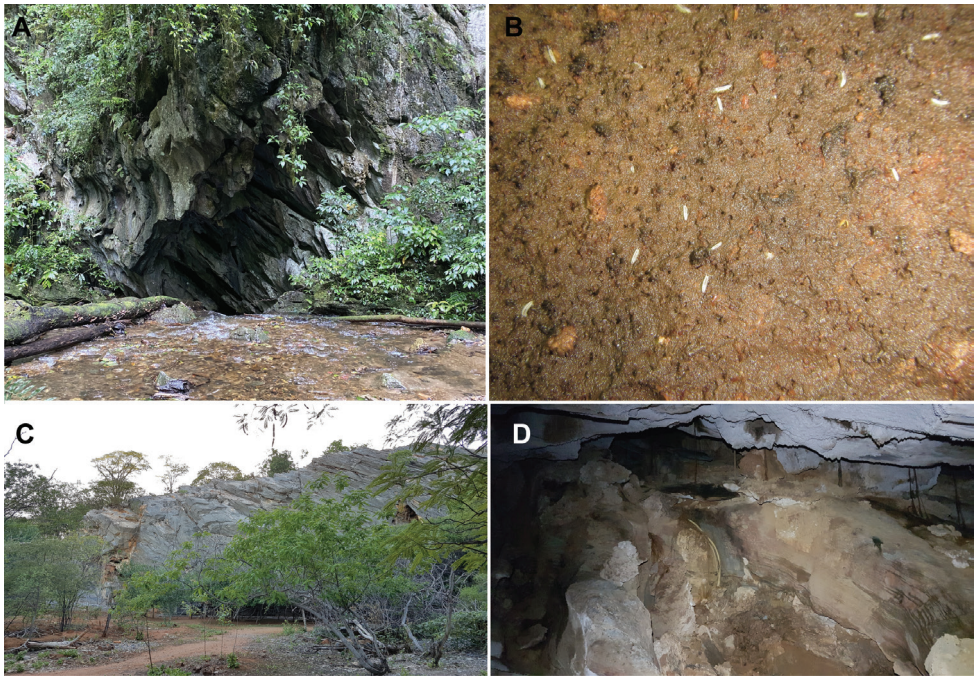


Figure 2. **A** Gruta da Tapagem (Caverna do Diabo), Açungui geomorphological group **B** *Cyldroniscus flaviae* Campos-Filho, Araujo & Taiti, 2017 foraging in the organic matter **C** surrounding area outside Gruta Toca Coroa do Frade, Casa Nova geomorphological group **D** aphotic zone of the Toca Coroa do Frade.

endopod inserted proximally. Male pleopod 1 endopod of two articles, distal article flagelliform. Male pleopod 2 endopod consisting of two articles, distal portion stout bearing complex apparatus.

Remarks. The genus *Pectenoniscus* was created by Andersson (1960) to allocate the new species *P. angulatus* from Itá, Nova Teutônia, state of Santa Catarina, Brazil. The author defined the genus by having the cephalon of “*Trichoniscus*-type”, dorsal surface of the cephalon and pereon with rounded tubercles, pleonites epimera large, left mandible with two penicils near lacinia mobilis, right mandible with one penicil near lacinia mobilis plus one in the molar process, maxillula outer endite composed of nine teeth and two slender stalks, inner endite of three penicils and proximal one longer than distal ones, genital papilla pear-shaped with tubelike termination, male pleopod 1 of “*Styлонiscus*-type”, male pleopod 2 endopod with distal portion broad and bearing a comb-like formation, and male pleopod 5 exopods with a dorsal lobe to fit the pleopod 2 endopod. Campos-Filho et al. (2019) described *P. liliae* Campos-Filho, Bichuette & Taiti, 2019 from Caverna Chico Pernambuco, Coribe, Serra do Ramalho karst area, state of Bahia, and added some characters in the diagnosis of the genus. Recently, Cardoso et al. (2020b) described six new species from karst areas of the states of Bahia and Minas Gerais, increasing the knowledge on the diversity of the genus, that now comprises eight species, i.e., *P. angulatus*, *P. carinhanhensis* Cardoso, Bastos-Pereira, Souza &



Figure 3. Gruna do Govi, Bambuí geomorphological group **A** surrounding area outside the cave **B** cave entrance **C,D** outside cave illustrating the anthropic impacts **E** *Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov. foraging in the organic matter **F** cave habitat where the specimens were collected.

Ferreira, 2020, *P. iuiensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *P. juvenilis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *P. liliae*, *P. montalvaniensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, *P. morrensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020, and *P. santanensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020.

***Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov.**

<http://zoobank.org/416BE93E-CA7C-4D98-9D6C-FE1BB265D264>

Figs 1, 3E, 4A-C, 5, 6

Material examined. BRAZIL • 1♂, **holotype**, Gruna do Govi, Feira da Mata, Serra do Ramalho karst area, Bambuí geomorphological group, state of Bahia, 13°56'43.30"S, 44°14'25.94"W, 12.X.2020, leg. ME Bichuette, DF Torres, JS Gallo, LS Horta and JE Gallão, LES 27761 • 1♂ (parts in micropreparations), **paratype**, same data as for holotype, LES 27762 • 2 ♀♀, **paratypes**, same data as for holotype, LES 27763.

Description. Maximum length: ♂ 2.2 mm, ♀ 3.5 mm. Dorsal surface slightly granulated, granules on pereonites 1–7 in two transverse rows, pleon smooth (Fig. 4A, B). Dorsal scale-setae tricorn-shaped (Fig. 5A). Cephalon (Figs 4C, 5B) with well-developed quadrangular antennary lobes, slightly directed outwards; profrons with suprantennal line bent downwards medially. Pereonite 1–3 epimera with postero-lateral corners right-angled, 4–7 progressively more acute and directed backwards; pleonite 5 epimera with glandular pores at sides near distal margins (Figs 4A, B, 5C). Telson (Fig. 5C) almost three times as wide as long, with concave sides and rounded apex. Antennula (Fig. 5D) with distal article longer than

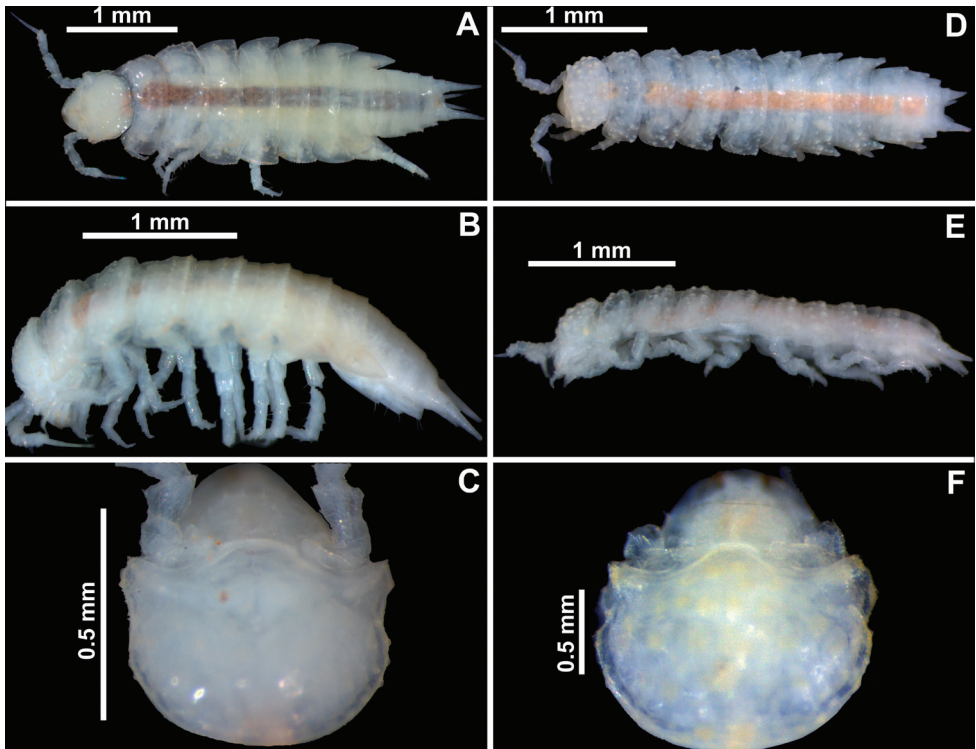


Figure 4. *Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov. (♀, LES 27763) **A** habitus, dorsal view **B** habitus, lateral view **C** cephalon, dorsal view. *Pectenoniscus fervens* Campos-Filho, Taiti & Bichuette, sp. nov. (♀, LES 27764) **D** habitus, dorsal view **E** habitus, lateral view **F** cephalon, dorsal view.

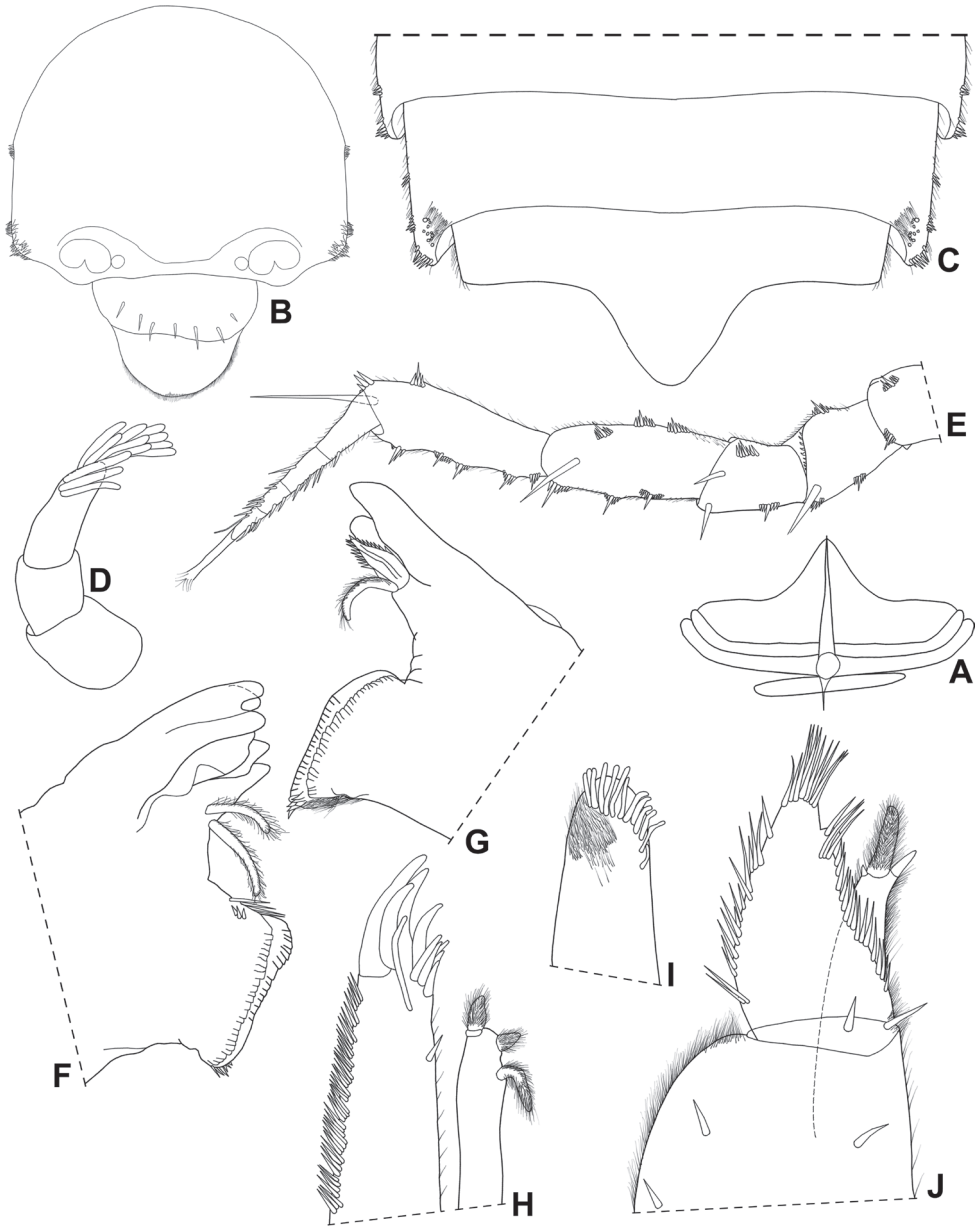


Figure 5. *Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov. (♂, LES 27762) **A** dorsal scale-seta **B** cephalon, frontal view **C** pleonites 4, 5 and telson **D** antennula **E** antenna **F** left mandible **G** right mandible **H** maxillula **I** maxilla **J** maxilliped.

second and first, and bearing at least 12 aesthetascs. Antenna (Fig. 5E) with fifth article of peduncle as long as flagellum, bearing one distal strong seta; flagellum of four articles, first article longest, apical organ as long as distal article of flagellum.

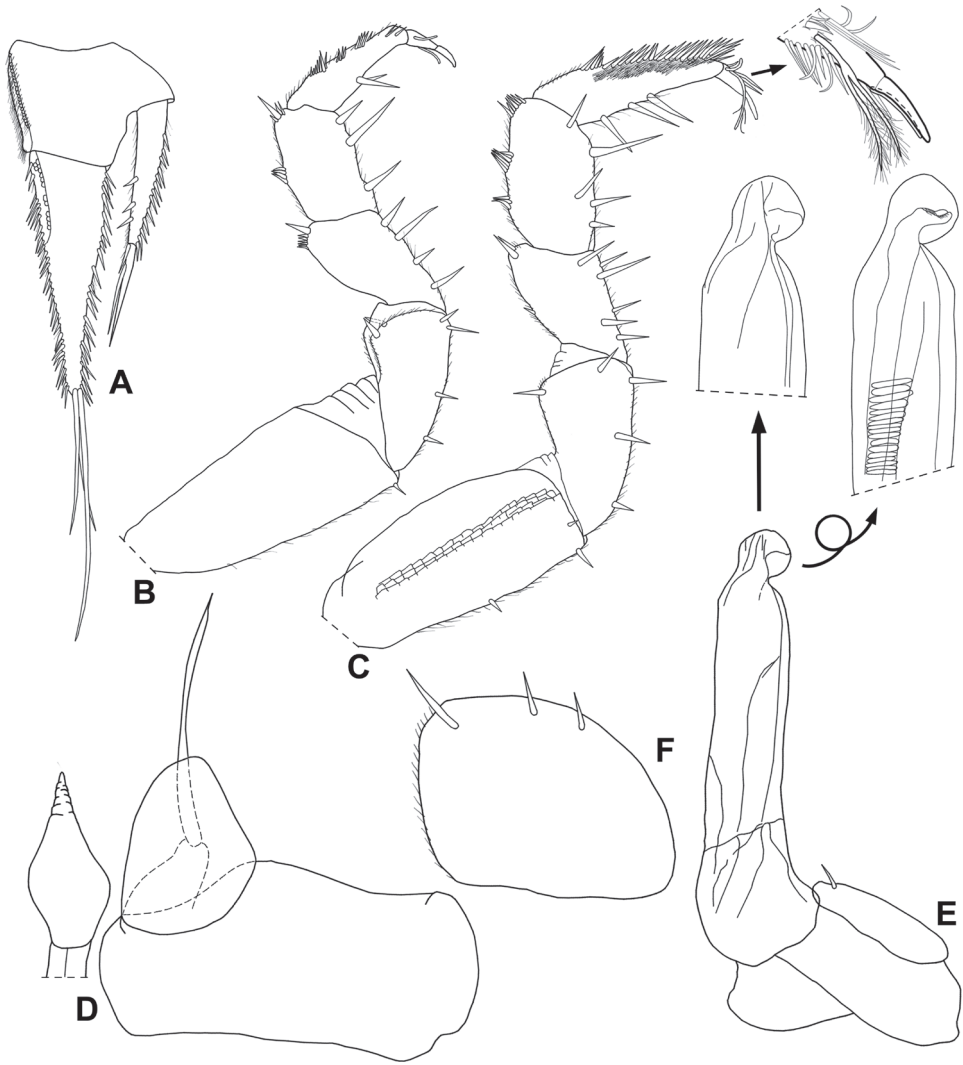


Figure 6. *Pectenoniscus pankaru* Campos-Filho, Torres & Bichuette, sp. nov. (♂, LES 27762) **A** uropod **B** pereopod 1 **C** pereopod 7 **D** genital papila and pleopod 1 **E** pleopod 2 **F** pleopod 5 exopod.

Mandibles as in Fig. 5F, G, right mandible with leaf-like lacinia mobilis. Maxillula (Fig. 5H) with two robust penicils; outer endite with 4+5 teeth, apically simple, one subapical slender stalk near medial margin. Maxilla as in Fig. 5I. Maxilliped (Fig. 5J) basis with lateral sides fringed with fine setae; palp with first article bearing two setae, distal articles fused and bearing many setae on lateral margins; endite much longer than wide, lateral margins covered with fine setae, distal margin bearing two strong setae and one elongated penicil. Grooves and scales for water conducting system on ischium, merus, carpus and propodus of pereopod 6 and basis of pereopod 7 (Fig. 6C). Dactylus with unguinal seta simple and dactylar seta bifid and setose.

Uropod (Fig. 6A) protopod and exopod grooved on outer margins bearing glandular pores; exopod longer than endopod, endopod inserted proximally.

Male. Pereopods 1–6 (Fig. 6B) without any sexual modifications. Pereopod 7 (Fig. 6C) propodus with brush of setae on rostral margin. Genital papilla (Fig. 6D) enlarged on median portion, apical part narrow and elongated. Pleopod 1 (Fig. 6D) protopod subrectangular, distal margin sinuous; exopod subtriangular, outer margin almost straight, proximal and outer margins convex; endopod longer than exopod, basal article short, distal article three times longer than basal one. Pleopod 2 (Fig. 6E) exopod ovoid, more than three times as wide as long; endopod of two articles, thick-set, second article more than twice as long as first, distally bearing round shaped lobe directed outwards. Pleopod 5 exopod (Fig. 6F) subquadrangular, slightly wider than long, bearing three setae, distal margin rounded.

Etymology. The new species is named for the indigenous people Pankaru, who inhabited the northern portion of Serra do Ramalho region.

Remarks. *Pectenoniscus pankaru* sp. nov. differs from all other species of the genus in the shape of the male pleopod 2 endopod. Moreover, it differs in having the antennal flagellum composed of four articles (vs. five in *P. angulatus*, three in *P. carinhanhensis*, *P. iuiuensis*, *P. juveniliensis*, *P. lilae*, *P. montalvaniensis*, *P. morrensis* and *P. santanensis*), antennula bearing 12 aesthetascs (vs. six in *P. morrensis* and *P. santanensis*, eight in *P. angulatus* and *P. montalvaniensis*, nine in *P. juveniliensis* and *P. lilae*, ten in *P. iuiuensis*, and 11 in *P. carinhanhensis*) (see also Andersson 1960; Campos-Filho et al. 2019; Cardoso et al. 2020b).

Specimens of *Pectenoniscus pankaru* sp. nov. were found only in the aphotic zone associated to the sediment banks close to a small subterranean stream (Fig. 2B) and characterised by high humidity levels, which are more suitable for terrestrial isopods. The specimens were collected near vegetable debris.

***Pectenoniscus fervens* Campos-Filho, Taiti & Bichuette, sp. nov.**

<http://zoobank.org/59D8BAB6-221D-47ED-8CE0-519D8A16E5DE>

Figs 1, 2C, D, 4D, E, 7, 8

Material examined. BRAZIL●1♂ (parts in micropreparations), **holotype**, Gruta Toca Coroa do Frade, Coronel José Dias, Barra Bonita karst region, Casa Nova geomorphological group, state of Piauí, 8°47'51.58"S, 42°25'1.47"W, 8.I.2018, leg. DM Schimonsky, DF Torres and JE Gallão, LES 22421●6♀♀ (one with parts in micropreparations), **paratypes**, same data as for holotype, LES 27764.

Description. Maximum length: ♂ and ♀ 3 mm. Dorsal surface granulated, granules on pereonites 1–7 in two transverse rows, on pleonites 3–5 in one row (Fig. 4D, E). Dorsal scale-setae tricorn-shaped in middle segments (Fig. 7A). Cephalon (Figs 4F, 7B) with antennary lobes small, triangular and slightly directed outwards; profrons with suprantennal line bent downwards medially. Pereonites 1 and 2 epimera with postero-lateral corners rounded, 4–7 progressively directed backwards and more acute (Fig. 4D, E). Pleonites 3–5 epimera without glandular pores (Fig. 7C). Telson (Fig. 7C) twice as wide as long,

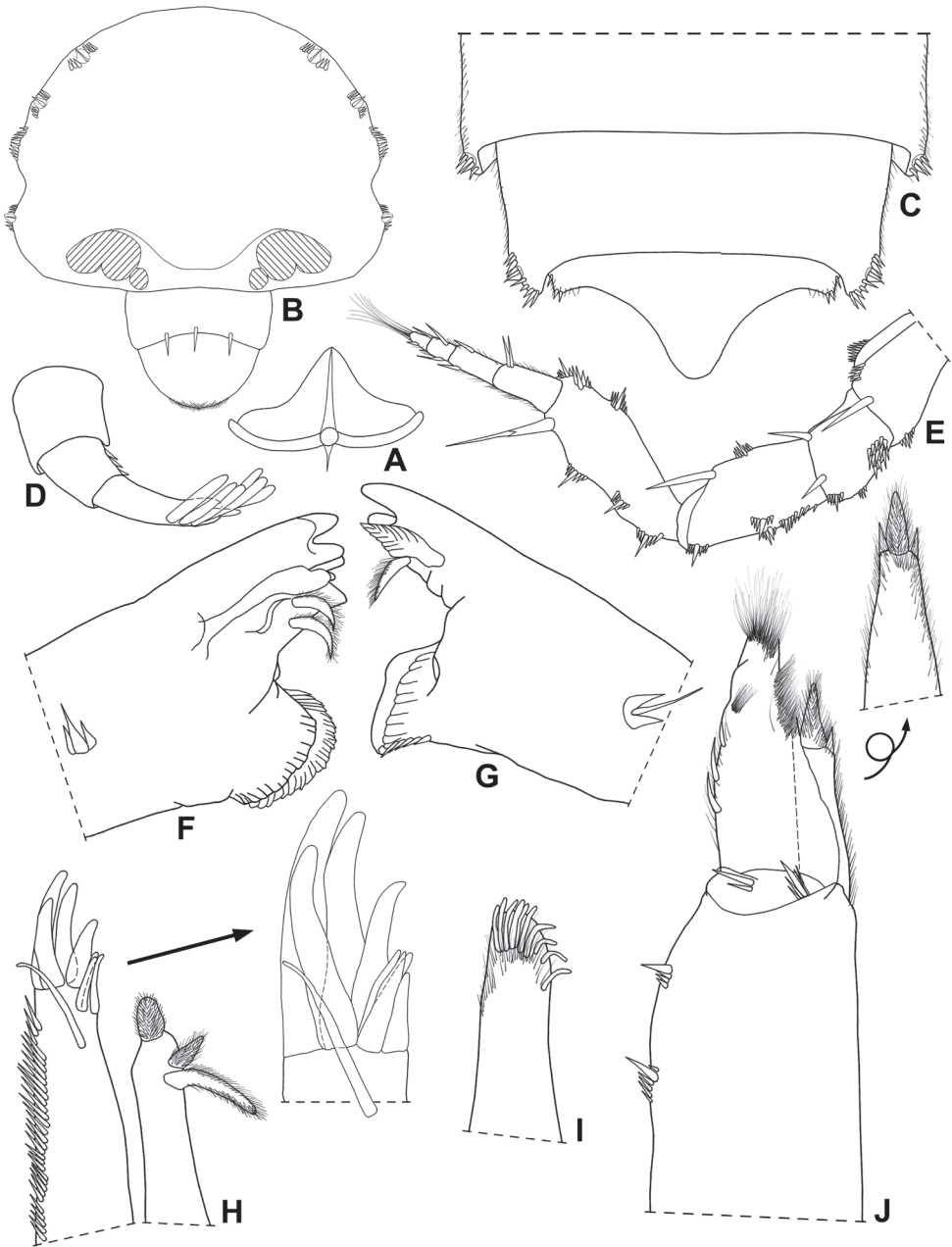


Figure 7. *Pectenoniscus fervens* Campos-Filho, Taiti & Bichuette, sp. nov. (♀, LES 27764) **A** dorsal scale-seta **B** cephalon, frontal view **C** pleonites 4, 5 and telson **D** antennula **E** antenna **F** left mandible **G** right mandible **H** maxillula **I** maxilla **J** maxilliped.

with concave sides and rounded apex. Antennula (Fig. 7D) with distal article longer than second and first, and bearing at least six aesthetascs plus distal tip. Antenna (Fig. 7E) with fifth article of peduncle slightly longer than flagellum, bearing one distal strong seta; fla-

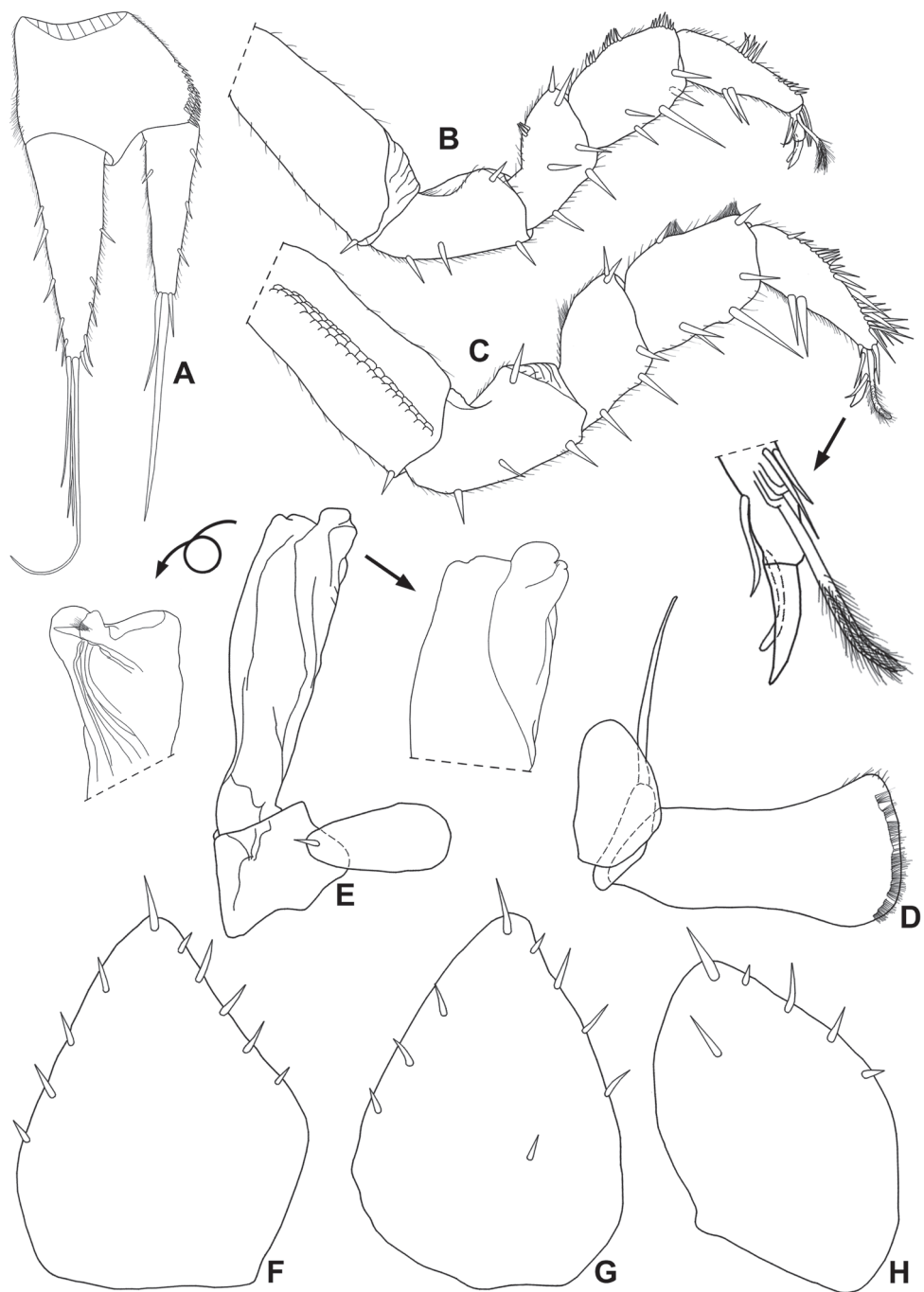


Figure 8. *Pectenoniscus fervens* Campos-Filho, Taiti & Bichuette, sp. nov. (♀, LES 27764) **A** uropod (♂, LES 22421) **B** pereopod 1 **C** pereopod 7 **D** pleopod 1 **E** pleopod 2 **F** pleopod 3 exopod **G** pleopod 4 exopod **H** pleopod 5 exopod.

gellum of four articles, first and second articles subequal in length, third and fourth articles shorter; apical organ longer than distal article of flagellum. Mandibles as in Fig. 7F, G; right mandible with leaf-like lacinia mobilis. Maxillula (Fig. 7H) inner endite with apical penicil robust; outer endite of 4+5 teeth, apically simple, one subapical slender stalk near medial margin. Maxilla as in Fig. 7I. Maxilliped (Fig. 7J) basis with lateral sides fringed with fine setae; palp with first article bearing two setae, distal articles fused and bearing distal fringe of fine setae; endite much longer than wide, lateral margins covered with fine setae, distal margin bearing two strong setae and one elongated penicil. Grooves and scales for water conducting system on ischium, merus, carpus and propodus of pereopod 6 and basis of pereopod 7 (Fig. 8B). Dactylus with ungual seta simple and dactylar seta simple and apically setose. Uropod (Fig. 8A) protopod and exopod not grooved on sternal margin; exopod longer than endopod and inserted almost at same level.

Male. Pereopods 1–7 (Fig. 8B, C) without any sexual modifications. Genital papilla as in previous species. Pleopod 1 (Fig. 8D) protopod subrectangular, distal margin concave; exopod ovoidal, outer margin almost straight, proximal, inner and distal margins rounded; endopod longer than exopod, basal article short, distal article ca. twice longer than basal one. Pleopod 2 (Fig. 8E) exopod ovoidal, twice as wide as long, one seta on inner margin; endopod of two articles, thickset, second article more than three times longer than first, distal portion subquadrangular, distal outer margin with round shaped lobe directed outwards bearing one triangular process on ventral margin. Pleopod 3–5 exopods as in Fig. 8F–H.

Etymology. Latin. *fervens* for very hot, boiling hot. The new species name refers to the very hot temperatures of the Brazilian state of Piauí.

Remarks. *Pectenoniscus fervens* sp. nov. differs from all other species of the genus in the shape of the male pleopod 2 endopod. Moreover, it differs from *P. pankaru* sp. nov. in having the antennula bearing six aesthetascs, the dactylar seta stout and in the different shape of the male pleopod 1 and pleopod 3–5 exopods. The new species shows the same number of aesthetascs of the antennula as in *P. morrensis* and *P. santanensis*, from which it differs in the antennal flagellum composed of four articles (three in *P. morrensis* and *P. santanensis*), male pleopod 1 protopod with the distal margin concave (vs. straight in *P. morrensis*, almost straight in *P. santanensis*), male pleopod 4 exopod triangular (vs. subrectangular in *P. morrensis*, subquadrangular in *P. santanensis*), and male pleopod 5 exopod rhomboid and longer than wide (vs. triangular and as long as wide in *P. morrensis*, subquadrangular in *P. santanensis*).

The specimens of *Pectenoniscus fervens* sp. nov. were found only in the aphotic zone (Fig. 2J), in vegetable debris with clay and under rocks, where the humidity was higher than at the cave entrance.

Discussion

The new species of *Pectenoniscus* described here showed strict dependence on high humidity. Their occurrence in the caves present in semiarid regions, where the exter-

nal temperatures are high and the humidity is low, reinforce the idea that these caves are probably important refuges for these animals due to their favourable conditions. Moreover, both species show remarkable troglomorphism, such as absent body pigments and eyes, which reinforces the classification of both species as troglobites.

A preliminary evaluation of the conservation status of the new species described here was carried out following the IUCN (International Union of Conservation of Nature) classification. *Pectenoniscus pankaru* sp. nov. and *Pectenoniscus fervens* sp. nov. were classified as Critically Endangered (CR) by the criteria B2ab(iii). The surrounding areas of the caves (Gruta do Góvi and Gruta Toca Coroa do Frade) are impacted by deforestation and the remaining native vegetation is present only close to their entrances. The Serra do Ramalho karst area is historically threatened by agricultural activities and potential mining projects (Gallão and Bichuette 2018). Furthermore, the Gruta do Góvi is used to capture subterranean water for the consumption of the local people and has pastures close to the cave, while the Gruta Toca Coroa do Frade, despite being close to the Parque Nacional da Serra da Capivara, is out of its boundaries and it is threatened by mining activities and increasing urbanisation. Moreover, both caves are not protected by any law.

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Inventory of the terrestrial isopods in Belgium (2011–2020)

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Abstract

This data paper describes a recent and spatially complete inventory of the terrestrial isopods of Belgium between 2011 and 2020. During these 10 years every $10 \times 10 \text{ km}^2$ cell of the Universal Transverse Mercator (UTM) grid in Belgium (373 grid cells) was visited in search for terrestrial isopods. Inventories covered different habitat types in every grid cell such as forest, wetlands or stream sides, and urban areas. Most of the dataset records were obtained by hand-collection methods such as turning stones and dead wood, or by sieving litter and through casual observations. These inventories were carried out by specialists from Spinicornis, the Belgian Terrestrial Isopod Group. Their data is complemented with pitfall trap data from scientific projects and verified citizen science data collected via waarneming.be and observations.be from the same time period. This resulted in 19,406 dataset records of 35 terrestrial isopod species. All dataset records are georeferenced using the centroid of their respective $5 \times 5 \text{ km}^2$ UTM grid cell. The dataset is published as open data and available through the Global Biodiversity Information Facility (GBIF). Direct link to the dataset: <https://doi.org/10.15468/mw9c66>.

Keywords

Detritivores, distribution, Europe, Isopoda, Oniscidea, woodlice

Data published through

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Rationale

Soils are one of the most complex and poorly studied habitats on Earth (Decaëns 2010). Soil communities are a major component of global terrestrial biodiversity, covering at least one-quarter of the world's biodiversity (Decaëns et al. 2006) and contributing to a wide array of ecosystem functions such as nutrient cycling, carbon sequestration, plant growth, and water storage (Lavelle et al. 2006; Wall et al. 2012). Additionally, up to 80% of all terrestrial primary production directly enters the detrital food web (Moe et al. 2005) to be processed by soil organisms that can be considered as the engine of nutrient cycling worldwide. Studying soil biodiversity is challenging because of the high diversity, small size, and high abundance of the organisms (Eisenhauer et al. 2017). However, soil macro-fauna (organisms larger than 2 mm; Jefferey et al. 2010) are easier to study because of their larger size and better-known taxonomy, and soil macro-fauna can therefore serve as model organisms for soil biodiversity. An important macro-fauna group in soils are terrestrial isopods. They are highly abundant in terrestrial soils and significantly contribute to nutrient cycling (Wolters and Ekschmitt 1997; Paoletti and Hassall 1999; David and Handa 2010). At global level, terrestrial isopod diversity is poorly studied. However, the group is fairly well studied in parts of Western Europe, such as in Great Britain and Ireland (Gregory 2009) or the Netherlands (Berg et al. 2008). In Belgium, however, studies and inventories of terrestrial isopods are mainly fragmentary even though the first publications date back to the first half of the 19th century and the first checklist was already made in 1870 (De Smedt et al. 2018a). A summary of the distribution of terrestrial isopods in Belgium was made around the year 2000 (Wouters et al. 2000). This summary revealed big distribution gaps for several species, and some species had been clearly overlooked. It was not until 2014 that a complete survey of the terrestrial isopods in Belgium started by a newly founded terrestrial isopod research group in Belgium called “Spinicornis” (De Smedt et al. 2018a, 2020). Spinicornis set as a goal to make an inventory of the terrestrial isopods in every 10 × 10 km² cell of the UTM (Universal Transverse Mercator) grid in Belgium (373 grid cells). During the survey, four species were newly discovered in Belgium (*Eluma caelata* (Miers, 1877), *Philoscia affinis* Verhoeff, 1908, *Porcellio monticola* Lereboullet, 1853, and *Trichoniscus alemannicus* Verhoeff, 1917) and an updated checklist was published (De Smedt et al. 2018a). All fieldwork was completed in 2020 and resulted in an ecological atlas (i.e., De Smedt et al. 2020) covering all native and free-ranging species. The data collected by Spinicornis was complemented with citizen-science data from the nature observations websites waarnemingen.be and observations.be, which are hosted

by Stichting Natuurinformatie and managed by the nature organisations Natuurpunt and Natagora. This paper describes these data, collected between 2011 (just before *Spinicornis* was founded) up to and including 2020, when the atlas project was finished.

Taxonomic coverage

The dataset covers data from 35 native species of terrestrial isopods (order Isopoda, suborder Oniscidea) found in Belgium between 2011 and 2020. Dataset records for three multispecies are also included for which species identification was not possible based on photographs or for samples lacking males (for species that can only be identified to the species level based on male genital characteristics). There are 36 native species in Belgium (De Smedt et al. 2018a) and only one native species (i.e., *Miktoniscus patiencei* Vandel, 1946) was not detected between 2011 and 2020. Species only occurring in greenhouses were excluded, as they are not part of the native or naturalized fauna of the country. De Smedt et al. (2017) gave more information on these species and their presence in Belgium. Nomenclature follows De Smedt et al. (2018a).

Taxonomic ranks

Kingdom Animalia

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Order Isopoda

Suborder Oniscidea

Family Armadillidiidae, Cylisticidae, Ligiidae, Oniscidae, Philosciidae, Platyarthridae, Porcellionidae, Trachelipodidae, Trichoniscidae

Genera *Androniscus*, *Armadillidium*, *Cylisticus*, *Eluma*, *Haplophthalmus*, *Hyloniscus*, *Ligia*, *Ligidium*, *Metatrachoniscoides*, *Oniscus*, *Philoscia*, *Platyarthrus*, *Porcellio*, *Porcellionides*, *Porcellium*, *Trachelipus*, *Trichoniscoides*, *Trichoniscus*

Species: Table 1.

Common names: terrestrial isopods, woodlice

Geographic coverage

Belgium is a small country (ca 30,500 km²) in Western Europe. It has a short shoreline (ca 65 km) along the North Sea. The shoreline borders the Netherlands in the north and France in the south. In the east, Belgium borders Germany and the Grand Duchy of Luxembourg. Belgium has a very high population density (374 inhabitants per km²). The main land use types are agriculture, urban area, and forest, with respectively 44.2%, 21.5% and 19.7% of the land area (STATBEL 2021). Despite its

Table 1. Species and multispecies represented in the dataset with their number of dataset records (N_{rec}), percentage of the total amount of dataset records (%), number of UTM 5 × 5 km² squares in which the species was recorded (N_{SQ}) and rank (R: 1 most squares, 35: least squares).

Species	Total	N_{rec}	(%)	NSQ	R
	18,572				
<i>Androniscus dentiger</i> Verhoeff, 1908		288	1.55%	128	15
<i>Armadillidium album</i> Dollfus, 1887		2	0.01%	1	35
<i>Armadillidium nasatum</i> Budde-Lund, 1885		518	2.79%	246	8
<i>Armadillidium opacum</i> (C. Koch, 1841)		176	0.95%	86	19
<i>Armadillidium pictum</i> Brandt, 1833		133	0.72%	58	24
<i>Armadillidium pulchellum</i> (Zencker, 1798)		116	0.62%	57	25
<i>Armadillidium vulgare</i> (Latreille, 1804)		1,927	10.38%	485	5
<i>Cylisticus convexus</i> (De Geer, 1778)		39	0.21%	10	29
<i>Eluma caelata</i> (Miers, 1877)		13	0.07%	4	33
<i>Haplophthalmus danicus</i> Budde-Lund, 1880		253	1.36%	136	14
<i>Haplophthalmus mengii</i> (Zaddach, 1844)		87	0.47%	76	20
<i>Haplophthalmus montivagus</i> Verhoeff, 1941		179	0.96%	138	13
<i>Hyloniscus riparius</i> (C. Koch, 1838)		204	1.10%	110	16
<i>Ligia oceanica</i> (Linnaeus, 1767)		48	0.26%	10	29
<i>Ligidium hypnorum</i> (Cuvier, 1792)		714	3.84%	301	7
<i>Metatrichoniscoides leydigii</i> (Weber, 1880)		27	0.15%	17	27
<i>Oniscus asellus</i> Linnaeus, 1758		3,322	17.89%	816	2
<i>Philoscia affinis</i> Verhoeff, 1908		184	0.99%	69	22
<i>Philoscia muscorum</i> (Scopoli, 1763)		2,927	15.76%	738	3
<i>Platyarthrus hoffmannseggii</i> Brandt, 1833		310	1.67%	164	9
<i>Porcellio dilatatus</i> Brandt, 1833		40	0.22%	12	28
<i>Porcellio laevis</i> Latreille, 1804		5	0.03%	2	34
<i>Porcellio monticola</i> Lereboullet, 1853		32	0.17%	7	31
<i>Porcellio scaber</i> Latreille, 1804		4,127	22.22%	853	1
<i>Porcellio spinicornis</i> Say, 1818		1,060	5.71%	492	4
<i>Porcellionides pruinosus</i> (Brandt, 1833)		171	0.92%	90	18
<i>Porcellium conspersum</i> (C. Koch, 1841)		106	0.57%	62	23
<i>Trachelipus rathkii</i> (Brandt, 1833)		367	1.98%	159	10
<i>Trichoniscoides albidus</i> (Budde-Lund, 1880)		154	0.83%	99	17
<i>Trichoniscoides helveticus</i> (Carl, 1908)		38	0.20%	30	26
<i>Trichoniscoides sarsi</i> Patience, 1908		95	0.51%	70	21
<i>Trichoniscus alemannicus</i> Verhoeff, 1917		18	0.10%	5	32
<i>Trichoniscus provisorius</i> Racovitza, 1908		182	0.98%	151	11
<i>Trichoniscus pusillus</i> Brandt, 1833		516	2.78%	307	6
<i>Trichoniscus pygmaeus</i> Sars, 1898		194	1.04%	140	12
Multispecies	834				
<i>Haplophthalmus danicus/mengii/montivagus</i>		59	7.07%	48	
<i>Trichoniscoides sarsi/helveticus</i>		44	5.28%	41	
<i>Trichoniscus pusillus/provisorius/alemannicus</i>		731	87.65%	367	
Total	19,406				

small size, Belgium has a rich geology ranging from a flat Atlantic region in the west, consisting of Holocene and Pleistocene deposits, to a more continental hilly landscape with Mesozoic and Pleistocene deposits in the east and the south. The Atlantic region has heavy clay soils in the northwest and loam and sandy loam soils in the central region (Fig. 1) with a high terrestrial isopod diversity (Fig. 2). On the sandy soils in the northern part of the Atlantic region, terrestrial isopod diversity is rather low (Fig. 2). Loamy soils make the connection between the Atlantic and the Continental region.

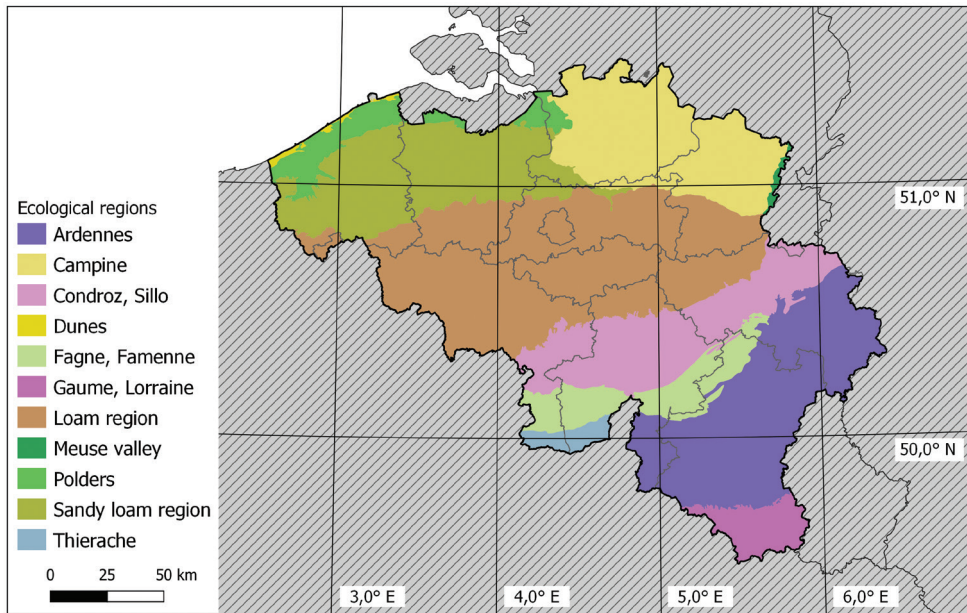


Figure 1. Map of Belgium indicating the different ecological regions. These ecoregions are ecologically defined areas with distinct assemblages of fauna and flora. The 11 ecological regions of Belgium are useful for analyzing terrestrial isopod diversity in Belgium (see De Smedt et al. 2020).

The Continental region has chalky soils in the central area and in the south (Fig. 1) and has a high species richness of terrestrial isopods (Fig. 2). In between these chalky soils and in the east of the country, slate and sandstone soils are present (Fig. 1) which are more species poor (Fig. 2). Because of this rich geology, Belgium has a diverse terrestrial isopod fauna containing both Atlantic (e.g., *Trichonicoides sarsi* Patience, 1908 and *Trichonicoides albidus* (Budde-Lund, 1880)) and continental species (e.g., *Porcellium conspersum* (Brandt, 1833) and *Trichoniscus alemannicus* Verhoeff, 1917).

Bounding box

49°27'0"N and 51°32'24"N Latitude; 2°28'12"E and 6°27'36"E Longitude

Georeferencing method

The ObsMapp application (<https://observation.org/apps/obsmap/>), developed by volunteers in collaboration with Stichting Natuurinformatie and widely used by citizen scientists in Belgium, was used to record the terrestrial isopod observations for the inventory. ObsMapp is a smartphone application where you can record and annotate observations, add pictures in the field, and make use of the GPS module in your cell phone for georeferencing

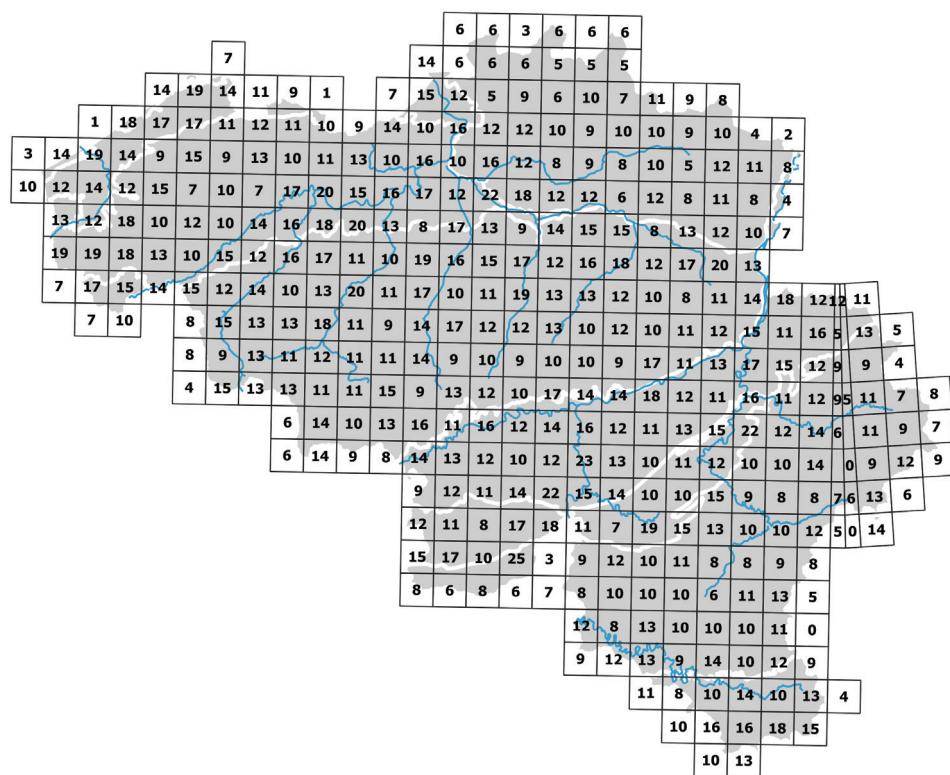


Figure 2. Number of terrestrial isopod species per $10 \times 10 \text{ km}^2$ Universal Transverse Mercator (UTM) grid cell in Belgium.

(Vercayie and Herremans 2015). The complemented data from Natuurpunt and Natagora were recorded through the waarnemingen.be and observations.be websites – both using the waarnemingen.be database – or by using the apps linked to the waarnemingen.be database (ObsMapp, iObs, Obsidentify). Original point locations were recorded, and they can be requested via natuurdata@natuurpunt.be. The observation data in the waarnemingen.be database was attributed to a $10 \times 10 \text{ km}^2$ grid cell for the original inventory and to a $5 \times 5 \text{ km}^2$ UTM grid cell for data publication purposes. The centroids of the $5 \times 5 \text{ km}^2$ grid cells were calculated using the WGS84 projection with a coordinateUncertaintyInMeters of 3,769 m. This uncertainty is the smallest circle that covers a complete UTM $5 \times 5 \text{ km}^2$ -square, as data inserted in GBIF are transferred from squares to circles Wieczorek et al. 2004). The total number of dataset records per $5 \times 5 \text{ km}^2$ grid cell is presented in Figure 3.

Temporal coverage

The dataset incorporates all records from 2011 (start 2011-01-01) up to and including 2020 (end 2020-12-31): a period of exactly 10 years. Across this period, total dataset records per month ranged from 1,114 in January to 2,553 in November (Fig. 4). During

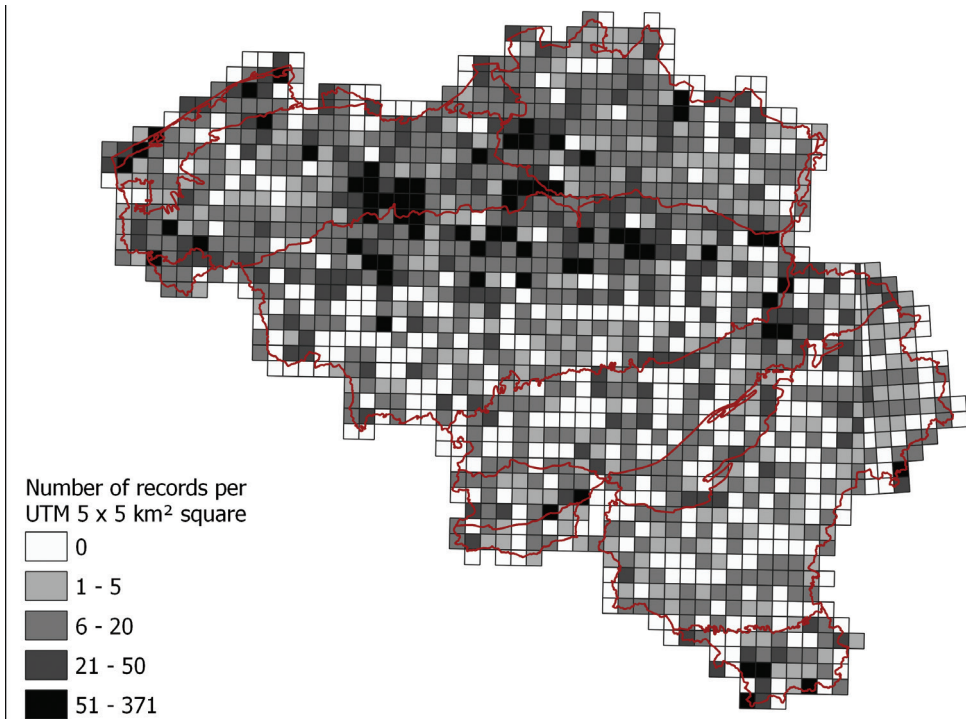


Figure 3. Number of dataset records per 5 × 5 km² Universal Transverse Mercator (UTM) grid cell in Belgium. Red lines delineate ecological regions.

the inventory period, there was a clear increase in number of dataset records since 2015, when Spinicornis was founded, with on average 2,919 (± 657) dataset records per year since 2015 and only 478 (± 266) dataset records per year between 2011 and 2014 (Fig. 5).

Observers

1,275 observers contributed to the dataset, of which 606 contributed more than one dataset record and 230 contributed more than five dataset records (Table 2). This makes the number of observations per observer highly skewed (Table 2). The five observers with the largest amount of dataset records (four personal accounts and one group account), all core members of Spinicornis, represent 12,236 dataset records (or 63% of the dataset).

Methodology

Sampling methods

Terrestrial isopod distribution data was collected in three ways: i) field observations during field excursions and subsequent microscopic species identification (for smaller

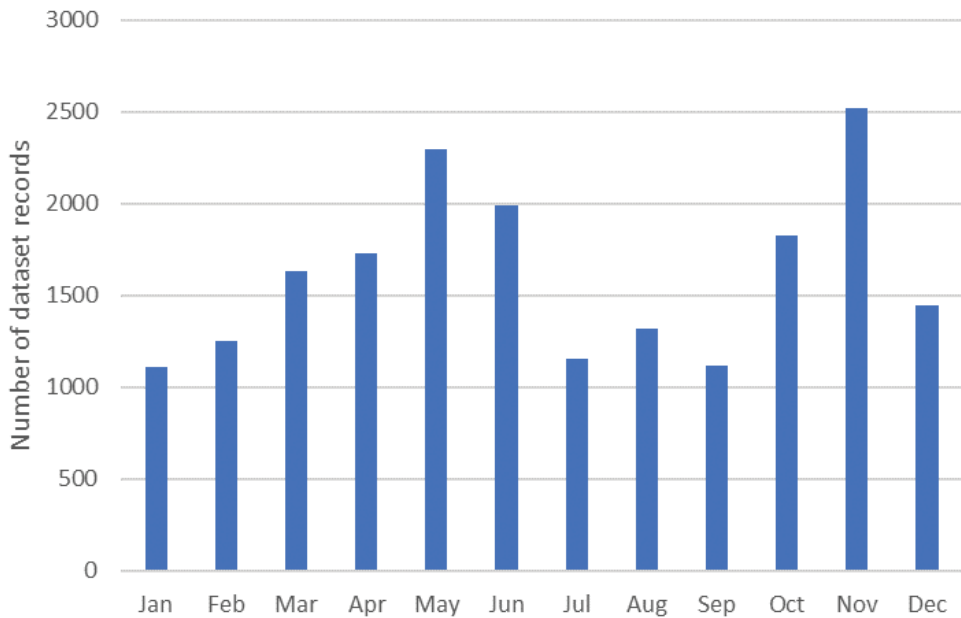


Figure 4. Total number of dataset records of terrestrial isopods per month across the inventory period (2011–2020).

species), ii) pitfall trap data from scientific projects assessing terrestrial isopod community composition in different ecosystems, and iii) ad hoc citizen science observations (see below). For all dataset records, the dataset contains species ID, date, and location. Certain species can only be identified based on the male genitalia. If only females were found or the exact species could not be identified based on a photograph, the species for this record is entered as a multispecies (i.e., *Trichoniscus alemannicus/provisorius/pu-sillus*, *Trichoniscoides helveticus/sarsi*, and *Haplophthalmus danicus/mengei/montivagus*).

The field surveys consisted of monthly field excursions in search for terrestrial isopods in different habitat types through hand collection by sieving litter and topsoil, as well as by turning stones, wood, etc. The UTM 10 × 10 km² grid was chosen as survey unit, balancing between efficient time investment and necessary resolution to create representative distribution maps. This resulted in a survey of 373 grid cells. In every grid cell at least three different habitat types were visited when the habitat type was present in the grid cell, i.e., (1) a forest and if possible, an ancient deciduous forest, (2) a streamside, riverbank or (wet) grassland, and (3) an anthropogenic habitat. Old quarries and coastal habitat were also searched if present in the grid cell. Graveyards were the preferred anthropogenic habitats due to their easy access and omnipresence but also a number of public parks, (old) farms, and allotment gardens were visited. These habitat types cover the core habitat of all terrestrial isopod species in Belgium. Every grid cell was visited between September 2014 and February 2020. On every excursion at least one but mostly

Table 2. Number of observations according to the number of users in the dataset.

Number of Observations	Number of users
1	669
2–5	376
6–10	102
11–50	104
51–200	16
201–1000	3
>1000	5

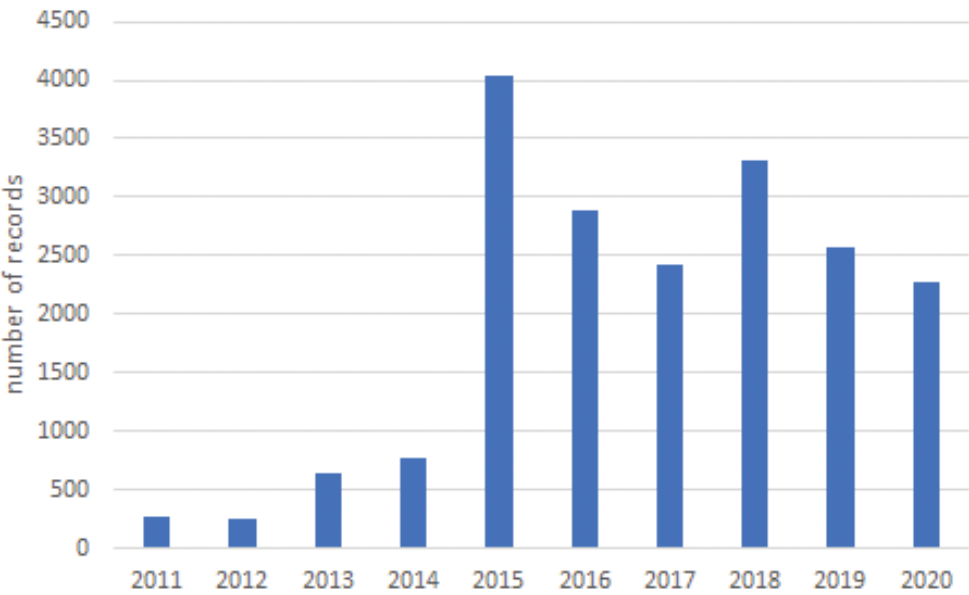


Figure 5. Total number of dataset records of terrestrial isopods per year across the inventory period (2011–2020).

three or four terrestrial isopod experts from Spinicornis were present to ensure quality control of the gathered data.

Pitfall trap data from scientific projects all originated from the northern part of Belgium (Flanders). The most important projects from which data was incorporated are Nijs et al. (2016), De Smedt et al. (2018b), Pardon et al. (2019), De Smedt et al. (2021), Perring et al. (2021), and the project “Future Floodplains” (www.futurefloodplains.be). All above-mentioned projects aimed to define the terrestrial isopod community composition in (semi-) natural habitat. Specific details can be obtained in the abovementioned papers. All individuals caught during these projects were identified by terrestrial isopod experts from Spinicornis.

Ad hoc citizen science data was collected via the nature observations websites waarnemingen.be and observations.be. The experts from Spinicornis used two methods to validate ad hoc citizen science data. The first and most important method con-

cerned validation of records for which the observer had attached clear pictures of the observed specimen. Only records with photographs enabling confirmed species identification were added to the dataset. The second method involved observations made by experienced volunteers. Dataset records were incorporated for a limited number of observers without photographs if observers made verifiable and correct identifications in the past.

Information withheld

Location information is generalized to 5×5 km² UTM grid cells. Observer name, exact XY-coordinates, toponyms, and photographs are not included in the published dataset, but are known in the source database.

Dataset

Dataset description

Inventory of the terrestrial isopods in Belgium (2011–2020) is an occurrence dataset published by Spinicornis (Belgian Terrestrial Isopod Group), the Research Institute of Nature and Forest (INBO), and Natuurpunt Studie. The dataset represents the most complete overview of terrestrial isopods in Belgium and includes occurrences of 35 species observed between 2011 and 2020. There are 36 native terrestrial isopod species in Belgium (De Smedt et al. 2018) and only one (i.e., *Miktoniscus patiencei* Vandel, 1946) has not been detected in Belgium between 2011 and 2020. The occurrences originate from field surveys, pitfall trap projects, and casual observations. The recorded data are registered through the citizen science portals waarnemingen.be and observations.be, which are managed by Natuurpunt Studie and Natagora, respectively. All data were verified by experts. Here, the dataset is published as a standardized Darwin Core Archive and includes for each record an occurrenceID, reference, date, location, and scientific name and, if available, also individual count, sex, lifestage, behavior, sampling protocol, and information on the identification.

The data are standardized to Darwin Core with a custom SQL view in the original waarnemingen.be database. They were published using the GBIF Integrated Publishing Toolkit (Robertson et al. 2014) instance at the INBO (<https://ipt.inbo.be>). The Darwin Core terms (<http://rs.tdwg.org/dwc/terms/>) in the dataset at the time of publication are: occurrenceID, type, language, license, rightsHolder, accessRights, references, datasetID, institutionCode, datasetName, basisOfRecord, informationWithheld, dataGeneralizations, individualCount, sex, lifestage, reproductiveCondition, behavior, occurrenceRemarks, samplingProtocol, eventDate, continent, countryCode, stateProvince, municipality, verbatimCoordinates, verbatimCoordinateSystem, verbatimSRS, decimalLatitude, decimalLongitude, geodeticDatum, coordinateUncertaintyInMeters, georeferenceRemarks, identificationVerificationStatus, identificationRe-

marks, taxonID, scientificName, kingdom, phylum, class, order, taxonRank, scientificNameAuthorship, vernacularName, nomenclaturalCode.

Technical description

Source publication: <https://ipt.inbo.be/resource?r=spiniicornis-occurrences>. This paper describes version 1.8 of this resource.

Dataset on GBIF <https://www.gbif.org/dataset/b6412a28-329c-4a24-b605-bc9d1b43b5b2>

DOI <https://doi.org/10.15468/mw9c66>

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First publication date 2021-02-24

Usage norms

To allow anyone to use this dataset, we have released the data to the public domain under a Creative Commons Zero waiver (<http://creativecommons.org/publicdomain/zero/1.0/>). We would appreciate, however, that these (<http://www.natuurpunt.be/normen-voor-datagebruik>) norms for data use are read and followed, and that a link is provided to the original dataset (<https://doi.org/10.15468/mw9c66>) whenever possible. If these data are used for a scientific paper, please cite the dataset following the applicable citation norms and/or consider us for co-authorship. We are always interested in providing more information and are available for help with analysing the data for your project, so please contact us via the contact information provided in the metadata or via pepijn@spiniicornis.be.

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Higher temperature and substrate vibrations as stress factors for terrestrial isopods – model species matter

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Abstract

This study was focused on behaviour of the Common Rough Woodlouse (*Porcellio scaber*) and the Plum Woodlouse (*Porcellionides pruinosus*) under the influence of stressors in the form of increased temperature, the vibrating surface, or their combination. Two types of experiments were performed. First, woodlice placed in a labyrinth were observed, to determine the degree of turn alternation, the speed of passing through the labyrinth, and the corrections of turn alternation, when exposed to stressors. In the second experiment how woodlice aggregate in the aforementioned potential stressors was recorded and whether the change in aggregation behaviour can be an indicator of the degree of stress. Increased temperature and the combination of increased temperature and vibrations were stressors only for *P. scaber*. The results show that vibrations are not a stress factor for *P. scaber* or *P. pruinosus*. *Porcellio scaber* passed through the labyrinth more slowly at increased temperatures, and although they made more turn-corrections, they alternated turns less intensely. Its aggregation behaviour was mainly influenced by temperature, which confirms that the aggregation behaviour of *P. scaber* actually indicates a degree of stress.

Keywords

Aggregation, Isopoda, Oniscidea, stress factor, turn alternation

Introduction

Humans are not the only ones to face stress. Although this may seem trivial from today's perspective, soil invertebrates such as isopods may also suffer from stress (Elwood et al. 2009). They have to solve existential problems to fulfil their necessities, such as the need for water or moisture (Cloudsley-Thompson 1956), food (Brody and Lawlor 1984), shelter (Allee 1926), and mating (Sutton 1972). This all takes place at constant risk of predation, either by one's own kind (cannibalism) (Sutton 1972) or by other species such as spiders (Gorvett 1956), ants, birds, amphibians, and mammals (Hegarty and Kight 2014). If isopods suffer from a deficiency or excess of any of these factors or the risk of predation, their behaviour may be affected (Sutton 1972). Just like vertebrates, invertebrates respond to stress caused by changes in the environment with a stress reaction. Isopods secrete substances into the haemolymph which are similar to glucocorticoids that can be found in vertebrates (Elwood et al. 2009). Stress can also shorten the maternal care period of the clutch (Kight and Nevo 2004). Isopod response to specific stimuli in nature is influenced by the mutual effect of distinctly intense individual stimuli and also by the current physiological state of isopods (Sutton 1972).

In the present study, we examined two types of defence behaviour of isopods, namely systematic turn alternation and the formation of an aggregation. A tendency to alternate turns is a behaviour known for different organisms including humans. Turn alternations are characterised by two types of reactions. A spontaneous reaction (Richman et al. 1986) is based on environmental stimuli, such as rodents' responses to a new maze (Montgomery 1952), or the reaction of cockroaches to a change in luminosity in a maze (Wilson and Fowler 1976). The second type of turn alternations is caused by the body's internal physiological response (Hughes 1989). Isopods are the most researched group for turn alternation patterns (Hughes 1967, 1978). When facing obstacles, isopods turn in opposite directions to create a deviation from linear motion (Hughes 1989) without being forced to do so by other external factors (Dingle 1965). Turn alternation patterns are probably caused by internal reactions to foot movements (Beal and Webster 1971). Several studies regarding this topic have been carried out (Kupfermann 1966; Hughes 1967, 1978, 1985, 1987, 1989, 2008; Moriyama 1999). Turn alternation pattern enables an escape from places with unfavourable conditions, such as lack of food (Hughes 1978), vibrations (Houghtaling and Kight 2006), the presence of predators (Carbines et al. 1992), and dehydration (Hughes 1967). Thus, turn alternations can serve as an indicator of stress. It is known that terrestrial isopods increase turn alternations in unfavourable environmental conditions to escape effectively, but previous habituation to disturbance can significantly reduce the stressor's effect (Houghtaling and Kight 2006). The extent of stress impact on turn alternation patterns depends on how long the isopods will be exposed to stress factors, i.e., how isopods will accustom or acclimate to a given source of stress (Warburg 1964; Cloudsley-Thompson 1969). Cividini and Montesanto (2018a) investigated the effect of vibrations on the alternate turns of isopods. They observed the increase of turn alternations rate in adult individuals of *Armadillo officinalis* Dumeril, 1816 with

the presence of vibrations when compared to *Armadillidium vulgare* (Latreille, 1804). The ability to perceive and respond to substrate-transmitted vibrations, in conjunction with alternate turns, increases with age (Cividini and Montesanto 2018b). Animals are likely to interpret species-specific and non-specific substrate-borne stridulations as a source of potential danger (Cividini et al. 2020). Turn alternation as an antipredatory strategy of woodlice has been examined by many authors such as Carbines et al. (1992), Houghtaling and Kight (2006), Hegarty and Kight (2014), and Cividini and Montesanto (2018b).

The formation of aggregations can be considered as an evolutionary successful reaction to unfavourable temperature, water loss, or predator pressure (Broly et al. 2013). One of the main reasons is that the isopods forming the aggregation makes individuals lose less water and are thus much less affected by the lack of humidity of the environment. Another reason is the reduction of CO₂ production (Allee 1926). Aggregation is mainly affected by thigmotaxis, attraction by individuals of the same species (Devigne et al. 2011), or by negative phototaxis. According to Allee (1926), there are two main types of grouping. True aggregation represents the stacking of individuals' bodies on top of each other with strong cohesion. More diffuse aggregations are typical by the lower number of individuals, higher mobility, and shorter length of contacts. Cividini and Montesanto (2018c) investigated the effect of vibrations on aggregation rates in *A. officinalis* and *A. vulgare*. Consistent with their previous work (Cividini and Montesanto 2018a), they found that *A. officinalis* responded to vibrations significantly and avoided zones of higher vibration intensity. Their ability to form large aggregations was lowered probably due to a reduced ability to find other individuals. In comparison to a sample of individuals with the absence of vibrations, they formed a large number of small aggregations. Even though aggregation behaviour in woodlice was examined in many works (Broly et al. 2013, 2014; Broly and Deneubourg 2015; Pogson 2016), understanding about the impact of aggregation on predation in terrestrial isopods is still relatively weak.

Despite turn alternations, Cividini and Montesanto (2018c) found that stressing conditions can alternate the aggregation behaviour of some terrestrial isopods too. Thus, the goal of this study was to test this claim on different species of terrestrial isopods and environmental stimuli. We evaluated the level of stress of two species exposed to substrate microvibrations, increased temperature, or their combination using turn-alternation in a T-maze. We assumed that both factors are stressful for isopods. Next, we tried to analyse the level of stress on aggregation behaviour under the same conditions.

Materials and methods

Porcellio scaber Latreille, 1804 (9–14 mm length) were hand-picked in an urban area of the village Bučovice, while *Porcellionides pruinosus* (Brandt, 1833) (3–8 mm length) were collected from a garden compost in the town of Hodonín. Woodlice were placed in 17 × 17 × 8 cm plastic boxes with a thin layer of plaster to maintain humidity, with

egg cartons used as an underlay. The plaster was kept moist, and isopods were fed on carrots *ad libitum*. Animals were kept in constant darkness with a temperature of 18–20 °C.

The experiment

The behavioural reaction to two stress factors or their combination was observed in both species. The first factor was increased temperature (27–31 °C, treatment coded as T+) while normal temperature (18–24 °C, called lower temperature and coded at T-) was used as a control. The second factor was the presence of microvibrations (coded as V+) and the absence of vibrations served as a control (V-). The experiments were carried out from August to October 2020. Before the beginning of each experiment, woodlice were exposed to a specific combination of conditions (T+V+, T+V-, T-V+, or T-V-, respectively) for two hours.

Two speakers with a power of 5 W were used to test the responses of the isopods to non-specific vibrations. There was a chipboard plate on the top of the speakers. The vibrations were produced by an artificially created recording, the same as the recording used by Cividini and Montesanto (2018a, b, c). The sound was played on an MP3 player connected to the speakers. Vibrations were measured with an oscilloscope application from a mobile phone placed of a T-maze between speakers, using the VibSensor application. RMS values of vibrations during minute measurement were X: (0.03); Y: (0.04); Z: (0.055) m/s².

To measure alternating turn behaviour, we used a plastic T-maze of size 15 × 11 cm consisting of two parts, i.e., the bottom labyrinth part and the cover with a small hole that served as an entry spot for subjects. Isopods were placed into the maze with entomological forceps, and then we observed how they alternate turns when trying to reach one of the six possible ending points. To prevent that woodlouse follow conspecific cues left by the previous woodlouse, the plastic labyrinth was placed on white A4 paper which was replaced after each trial. We also recorded changes in isopod's turn decision (isopod made U-turn and used opposite corridor), and the time needed for reaching one of the end points of the labyrinth (Fig. 1). If woodlice did not start to move within 3 min, trial was terminated. To simplify the calculation of turn alternation pattern, a specific value was assigned to each end point of the labyrinth (depending on how

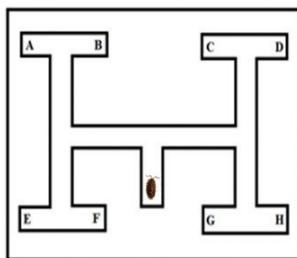


Figure 1. Scheme of T-maze (labyrinth) used for evaluation of turn alternations.

many alternating turns isopod made). Reaching the points A or D with assigned value 1 meant that turn alternation pattern was systematic, i.e., turn left-right-left (L-R-L) or turn right-left-right (R-L-R, see Fig. 1). Points B and C had a value of 1.6 because animals started turn alternation pattern but did not complete it (turns L-R-R, or R-L-L). Ends E and H with a value of 2.3 indicated that isopods did not start with a turn alternation pattern from the beginning but only after the second turn (turns L-L-R, or R-L-L). Reaching the points F and G with the value of 3 was considered the result of not systematic turn alternation (R-R-R or L-L-L).

To observe aggregation behaviour, 30 individuals of the same species were placed into a box, and recorded on camera for two hours. Before the experiment, the plaster inside each box was thoroughly moistened to provide enough humidity. For filming, a small Niceboy outdoor camera installed on a tripod was used. We analysed 12 images (one every 10 min) of each video and calculated the number of isopods touching each other, i.e., the presence of thigmotaxis. After filming, isopods were returned to the breeding boxes. The results are expressed as the average aggregation dynamics for all four variants of observation. In total, 46 aggregation dynamics of *P. scaber* and 49 aggregation dynamics of *P. pruinosis* were analysed.

Data analysis

For T-maze experiments, we analysed the level of turn alternations according to the end point, the time needed for reaching the end point, and the number of changes in turn alternation. For aggregation experiments, the number of aggregated animals (individuals in contact) every ten min were analysed. All results were evaluated using a one-way ANOVA with a significance level of $\alpha = 0.05$. The presence of vibrations with the increased temperature was coded by the number 1, while the absence of both factors was marked as 0. Pearson's correlation test was used to evaluate the dependence of the turn alternation and the speed of passage through a maze.

Results

Turn alternation

Three behavioural characteristics of movements were examined in the maze. The first was the rate of a random ramble (negatively correlated with turn alternation). The second variable was the time spent in the labyrinth, measured from the entry of an isopod into the maze until it reached one of the possible end points. The third variable was the extent of changes in turn alternation pattern, i.e., the number of returns and changes in the turn alternation in the labyrinth. A total of 280 individuals of *P. scaber* and 301 individuals of *P. pruinosis* were tested in this type of experiment.

Our results did not show a statistically significant association between the rate of a random/unspecific ramble (reversed value of systematic turn alternation) and the

presence of microvibrations ($F = 0.09$; $p = 0.761$) for *P. scaber*. The average rate of a random ramble for the presence of vibrations was 1.74 and for the absence of vibrations was 1.71. There was no significant effect of vibrations on the time spent in the labyrinth ($F = 1.45$; $p = 0.229$), although the individuals of *P. scaber* exposed to vibrations ran through the labyrinth with an average time of 38 sec vs. 45 sec with the absence of vibrations. Also, there was no significant association between the presence of vibrations and changing of the turn alternation pattern for this species ($F = 0.20$; $p = 0.657$). An average number of changes during the presence of vibrations was 0.87 in contrast with 0.77 during their absence.

In contrast, for *P. pruinosis*, the association between the rate of a random ramble and the presence of microvibrations was statistically significant ($F = 5.01$; $p = 0.026$). The average rate of a random ramble during the presence of vibrations was 1.86 and during their absence was 1.67. Isopods made more systematic turn alternation with the absence of vibrations. There was no significant effect of vibrations on the length of the time spent in the labyrinth ($F = 0.03$; $p = 0.862$). The average time spent in the labyrinth with the presence of vibrations was 37 sec while with the absence of vibrations it was 38 sec. There was no significant association between the presence of vibrations and change of turn alternation ($F = 2.67$; $p = 0.103$). An average number of changes in turn alternation with the presence of vibrations was 1.13 and 0.74 when vibrations were absent.

In the case of *P. scaber*, we found out the significant associations between increased temperature and the rate of a random ramble ($F = 21.84$; $p < 0.001$). The average rate of random ramble during exposure to increased temperatures was 1.92, while at lower temperatures it was 1.52. Thus, this species made less alternating turns in an increased temperature environment. Results also showed a statistically significant association between the time spent in the labyrinth and increased temperature ($F = 30.65$; $p < 0.001$). Individuals exposed to increased temperatures ran through the labyrinth with an average time of 58 seconds while in lower temperatures it was 25 seconds. Thus, isopods spent more time in a maze when temperatures were increased. We also found a significant association between temperatures and changes in turn alternation pattern ($F = 25.56$; $p < 0.001$). An average number of changes of turn alternation during exposure to increased temperature was 1.33 in comparison to 0.30 at lower temperature. An increasing number of changes in turn alternation was observed in woodlice behaviour when the temperature was increased.

For *P. pruinosis*, the associations between increased temperature and the rate of a random ramble were not significant ($F = 0.02$; $p = 0.891$). The average rate of random ramble during exposure to increased temperatures was 1.76, while at lower temperatures it was 1.77. There was no significant association between the time spent in the labyrinth and increased temperature ($F = 0.79$; $p = 0.375$) for this species. The average time spent in the labyrinth was 35 seconds per individuals exposed to increased temperatures and 40 seconds per those exposed to lower temperatures. We prove the existence of a significant association between temperatures and change of turn alternation pattern ($F = 4.44$; $p = 0.036$). An average number of changes in turn alternation during exposure to increased temperature was 1.2; for lower temperatures it was 0.7. For *P. pruinosis*, the increased temperature significantly increased the number of changes in turn alternation pattern.

Combination of increased temperatures and the presence of vibrations has significant effect on rate of random ramble ($F = 8.99$; $p < 0.001$; Fig. 2a) for *P. scaber*. The highest average rate of the random ramble was at increased temperatures with the presence of vibrations (2.04) in opposite to lower temperatures with the presence of vibrations (1.47). Also, the effect of a combination of temperatures and vibrations on time spent in the labyrinth was statistically significant ($F = 15.80$; $p < 0.001$; Fig. 2c). *Porcellio scaber* ran through the labyrinth the fastest at lower temperatures with vibrations (13 sec) and the slowest at increased temperatures associated by vibrations (66 sec). The vibrations thus increased the isopod's speed while the increased temperature slowed them down. The effect of the combination of increased temperature and vibrations on changes in turn alternation was significant ($F = 9.38$; $p < 0.001$; Fig. 2e) for this species. In average, highest numbers of changes in the turn alternation were made at increased temperatures associated by vibrations (1.55 changes) compared to lower temperatures with presence of vibrations (0.24 changes). The presence of vibrations thus reduced the number of changes in turn alternation, while the higher temperature, on the contrary, increased them.

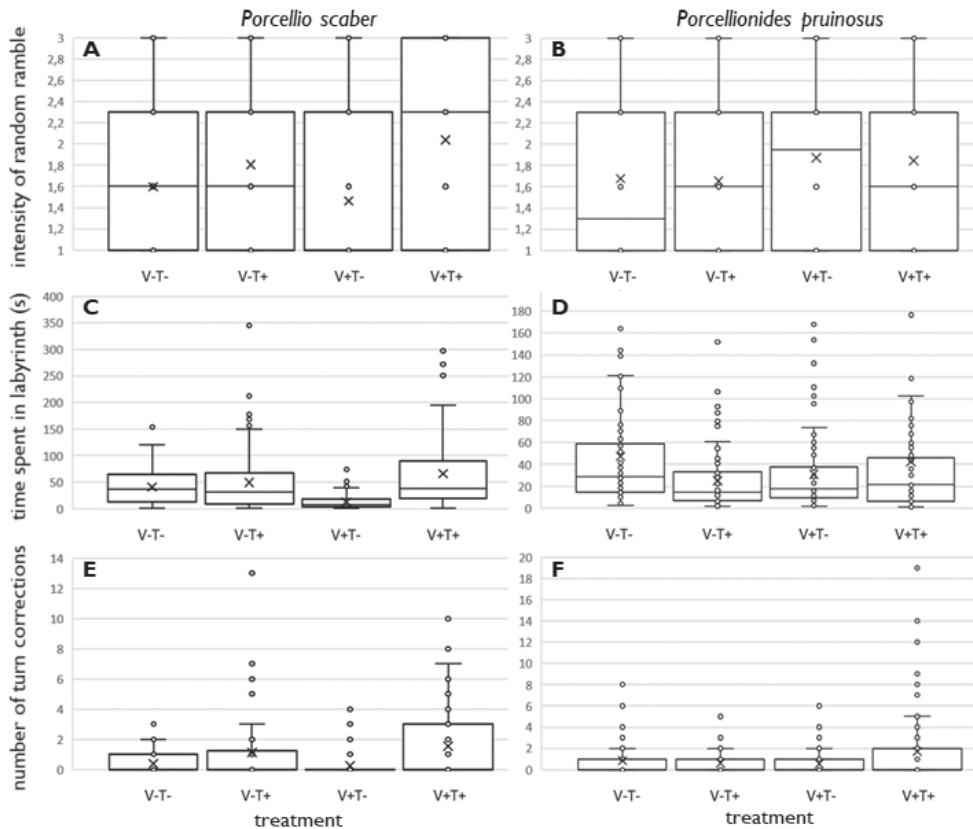


Figure 2. Characteristics of movement in T-maze of *P. scaber* (a, c, e) and *P. pruinosus* (b, d, f) at different treatments: a, b intensity of random/unspecific ramble, i.e., reversed value of systematic turn alternation c, d speed of passing through the labyrinth e, f number of self-corrective turns. Treatments V-/V+ mean absence/presence of substrate vibrations and T-/T+ mean low/high temperature.

For case of *P. pruinusosus*, combination of increased temperatures together with presence of vibrations has no significant effect on rate of random ramble ($F = 1.69$; $p = 0.170$; Fig. 2b). The highest average rate of the random ramble was at low temperatures along with the presence of vibrations (1.87) and the lowest at increased temperatures with the absence of vibrations (1.65). The effect of a combination of temperatures and vibrations on time spent in the labyrinth was statistically significant ($F = 3.34$; $p = 0.020$; Fig. 2d). Isopods ran through the labyrinth with the highest speed at increased temperatures with the absence of vibrations (25 sec) and the slowest at lower temperatures with the absence of vibrations (47 sec). We proved the existence of statistically significant effect of the combination of increased temperature, vibrations on the change of turn alternation ($F = 5.38$; $p = 0.001$, Fig. 2f). On average, highest numbers of changes in the turn alternation were made at increased temperatures combined with the presence of vibrations (1.71) and the lowest numbers when isopods were exposed to lower temperatures combined with the presence of vibrations (0.54).

We also found out a significant weak positive correlation ($R = 0.32$, $p < 0.00001$.) between the intensity of the turn alternation of *P. scaber* (i.e., the probability of running to one of the ends, indicating systematic turn alternation like A or D) and the speed of passage through the maze. When *P. scaber* ran slowly, there was higher probability that it will reach one of the “wrong” ends, which indicates unsystematic alternating turns. Results for *P. pruinusosus* show no correlation ($R = 0.06$, $p = 0.264619$) between the intensity of the turn alternation and the speed rate of passage through a maze.

Aggregation

Group of two or more woodlice in contact were considered to be an aggregate. The distributions of the individuals were determined by counting the number of aggregated individuals in each box every 10 min during the 120-min experiment.

For *P. scaber* the results showed that there is a statistically significant difference in aggregation dynamics of isopods exposed to vibrations ($F = 5.71$; $p = 0.003$). Fig. 3 shows that the isopods presented different aggregation dynamics depending on different temperatures. At a lower temperature, ~ 23–26 individuals (out of a total number of 30) were aggregated during the whole two hours of observation (Fig. 3). In comparison, at increased temperatures the aggregations were initially smaller (20 individuals) but increased within half an hour, and stabilised at the number ~ 25–28 individuals (Fig. 3). Aggregations on a vibrating surface were usually smaller than aggregations on a stable substrate.

In *P. pruinusosus*, vibrations together with increased temperature had a significant effect on the dynamic and size of aggregation ($F = 83.52$; $p < 0.001$). A higher number of aggregated individuals was observed among isopods exposed to lower temperatures combined with the presence of vibrations (after an hour, half of the total of 30 individuals were in aggregations). In comparison, numbers of aggregated isopods were the lowest at increased temperature with the absence of vibrations. In all of the observed variants it can be seen a slight increase in the number of aggregated individuals over time (Fig. 3). Aggregations tended to repeatedly appear and disappear.

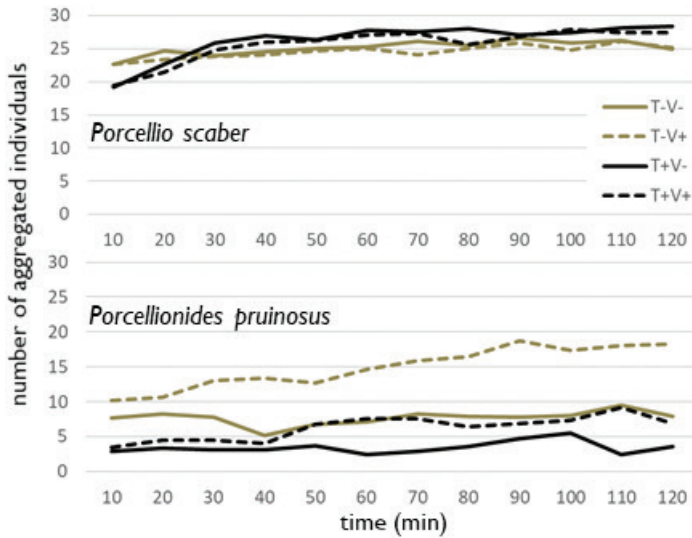


Figure 3. Dynamics of aggregations of *P. scaber* and *P. pruinosus* during 2-hr observations at different treatments. V-/V+ mean absence/presence of substrate vibrations and T-/T+ mean low/high temperature.

Discussion

Turn alternation

Our results showed that the effect of vibrations on the rate of turn alternation of *P. scaber* was not significant. When the substrate did not vibrate, *P. pruinosus* significantly increased turn alternations. For both species, the vibrations did not affect the time spent in the labyrinth or changed turn alternation pattern. This is probably because neither *P. scaber* nor *P. pruinosus* has any stridulatory or auditory organs to absorb vibrations. Those can be found in species like *A. officinalis* that is probably able to generate and receive vibrations (Cividini and Montesanto 2018a, 2020). The presence of mechanoreceptors that detect substrate vibrations in isopods is well documented (Zimmerman and Kight 2016), but the monotonous vibrations probably do not resemble an oncoming predator. The effect of monotonous and interrupted substrate vibrations on isopod behaviour should be studied in future studies.

A different effect of vibrations to turn alternations was reported by Moriyama et al. (2016), who observed woodlice at room temperature. In their study, approximately a quarter of the woodlice exposed to vibrations alternated their directions. Houghtaling and Kight (2006) proved that habituation to disturbance can significantly reduce turn alternations. Insignificant reaction to vibrations could be caused by the fact that before each experiment, woodlice were exposed to vibrations for a period of two hours. The animals could be used to the vibrations, thus the weak response during the experiment.

An increased temperature did not stress *P. scaber*, because isopods spent more time in the maze and alternated their turns less systematically. This result is unexpected

because it is contrary to the expectation based on the findings of Warburg (1964) and Schuler et al. (2011), who reported that at increased temperatures, *P. scaber* ran faster. It is also contradictory to the findings of Cloudsley-Thompson (1956), who mentioned that increased temperatures represent a stressful factor for woodlice, mainly due to its impact on water loss (causing their gradual drying and therefore triggering more movement). In contrast, there was no significant effect of increased temperature on the rate of a random ramble or on the time needed to complete the labyrinth in *P. pruinosus*. This species can be commonly found in compost or stacked bales of hay (Frankenberger 1959), which means they could be used to higher temperatures that compost goes through during intense microbial decomposition. *Porcellionides pruinosus* is more tolerant to increased temperatures showing a stable feeding rate for 20 °C as well as 28 °C than *P. scaber* is (Römbke et al. 2011). Meanwhile, *P. scaber* collected in Central Europe has a temperature optimum of 21 °C (Antol et al. 2019), indicating that increased temperature should be considered a stress factor for this species. After exposure to increased temperatures, both species made a higher number of returns or turn alternations. Hughes (1967) reported, that alternation of turns can be caused by the effort to escape from adverse conditions. Moriyama et al. (2016) found that ca. a quarter of the total number of tested woodlice made more returns or changes in the turn alternation pattern. The effect of increased temperatures was also mentioned by Warburg (1964), who found out that the *Oniscus asellus* Linnaeus, 1758 as well as *A. vulgare*, made more alternating turns after the exposure to increased temperatures. In our experiment, *P. scaber* ran slower and alternated turns less systematically at increased temperatures. This could be caused by the fact that before each experiment, isopods were exposed to the tested temperature for two hours. Khan and Khan (2008) reported loss of body mass of the water flea *Daphnia magna* Straus, 1820 in increased temperature resulting from previous hyperactivity. In similar fashion, *P. scaber* could be exhausted and therefore did not show a stronger activity. Also, Ferreira et al. (2016) stated that *P. pruinosus* shows signs of stress when exposed to temperatures above 30 °C, while to the temperatures below 20 °C it does not react at all. Refinetti (1984) and Nair et al. (1989) reported that *A. vulgare* and *Porcellio laevis* Latreille, 1804 can quickly acclimatise to increased temperature. Nevertheless, this may not apply for *P. scaber* from Central Europe, as both mentioned papers deal with (sub)tropical populations.

Vibrations, together with increased temperature, have a significant effect on the rate of random ramble, time spent in the labyrinth, as well as the change of turn alternation in *P. scaber*. These results have the same pattern as those with increased temperature alone. Apparently, vibrations were not stressful for *P. scaber*, probably due to its origin in the city environment. Houghtaling and Kight (2006) reported that urban isopods were adapted to microvibrations. Our results showed that the combination of temperature and vibrations, similar to increased temperature alone, did not affect turn alternation of *P. pruinosus*, potentially due to characteristics of the places they typically occur at. The increased temperature together with vibrations significantly affected changes in turn alternation. Changes in turn alternation were significantly affected by the higher temperature together with vibrations, as well as by the higher temperature alone.

Aggregations

Porcellio scaber showed a statistically significant difference in aggregation dynamics after their exposure to vibrations. At lower temperatures, somewhat stable aggregations of ~23–26 individuals were formed. At increased temperatures, within half an hour, the number of aggregated isopods increased to approximately 28 individuals and then stabilised. This is probably because the optimal temperature for *P. scaber* is 21 °C (Antol et al. 2019) (i.e., our experimental “lower temperature”), and because at colder conditions water loss does not occur as quickly as at increased ones (Cloudsley-Thomson 1956). Woodlice exposed to increased temperatures tend to lose water faster, so they start to aggregate to prevent water loss. Isopods that are in the upper layer of crowded individuals leave the group more often to look for a more suitable place due to the quicker water loss (Allee 1926).

The aggregation of *P. pruinosus* was significantly affected by vibrations along with increased temperatures. Isopods aggregated more when exposed to lower temperatures. In an hour after the exposure more than half of the individuals were aggregated. This is in agreement with results of Cividini and Montesanto (2018c) regarding *A. officinalis*. This is probably related to the finding of Allee (1926), who mentioned that woodlice form two types of aggregations, namely the bunching or true aggregation that is seen in *P. scaber* and crowding or a more diffuse grouping observed in *P. pruinosus*, depending on their mutual contact and interactions. The isopods aggregated less at increased temperatures than at the lower one. According to the findings of Allee (1926), when isopods were under conditions unfavourable to aggregation such as the exposure to low temperatures, the tendency to aggregate increased. However, the increased temperature was not a stressful factor for *P. pruinosus*, probably because this species is used to the increased temperatures. Numbers of aggregated isopods were slightly higher due to vibrations.

Porcellionides pruinosus aggregated in greater numbers during the presence of vibrations than during non-vibration treatment. The same results showed an experiment by Cividini and Montesanto (2018c), who tested the effect of vibrations on *A. officinalis* at room temperature (20 °C). In the non-vibration treatment, isopods quickly began to aggregate and formed a single stable aggregation. However, *A. officinalis* aggregated less with the absence of vibrations. Cividini and Montesanto (2018c) mentioned that isopods could create a sound by rubbing different parts of the body against each other when conglobated, which could evoke other individuals to stay inactive and do not aggregate. Although *P. pruinosus* is not able to stridulate, its antipredatory response is not volvation, only running away and looking for hiding place.

Based on our findings, the pairing model species – stressor can be further refined for more significant results. Further research should be aimed at how stressful various temperature ranges for different isopod species are. Due to the ability of woodlice to acclimate to substrate vibrations, the future use of vibrations in experimental studies is very problematic. Perhaps shorter experiments with low air humidity as a stressor can be less difficult as our knowledge about the demands of different species is sufficient. The ability of different isopod species to habituate to stress factors could also play a certain role in this matter and future studies of this topic are encouraged.

Conclusions

Our results showed that for *P. scaber* and *P. pruinosus*, vibrations are not a stressful factor. This may be related to the fact that tested individuals have been collected in an urban environment where road and rail transport is a permanent source of substrate microvibrations, and the isopods are used to it. The increased temperature was a stressor only for *P. scaber*. This species did not show any major response to increased temperature; it went through the labyrinth more slowly at increased temperatures, and although they made more changes, they alternated turns less intensely. This behaviour could be caused by previous too long exposition to experimental conditions. *Porcellionides pruinosus* was not stressed by the increased temperature, which is probably caused by their occurrence in composts, where the temperature is often increased due to intense microbial decomposition. The aggregation dynamics of *P. scaber* was affected by the increased temperature. Initially, at increased temperatures isopods aggregated less or formed more unstable aggregations than the control group, but later the aggregations were stable and slightly larger than in the control group. Thus, the aggregation behaviour of *P. scaber* shows certain degree of stress, but its interpretation is relatively complicated. We were not able to confirm that *P. pruinosus* was stressed by vibrations or temperature, so it was not possible to make the comparison of the aggregation behaviour and the degree of stress. These results suggest that factors that have been used as stressors for specific species in some studies cannot be automatically used as stressors for other terrestrial isopod species.

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Avoidance behaviour toxicity tests should account for animal gregariousness: a case study on the terrestrial isopod *Porcellio scaber*

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Abstract

Avoidance behaviour enables woodlice to escape suboptimal environmental conditions and to mitigate harmful effects of pollutants. However, several studies have shown that at least in some woodlice species the tendency to aggregate can lead to suboptimal responses as the between-conspecific attraction can outweigh the aversive stimuli. The present study evaluated the influence of gregariousness on the behaviour of *Porcellio scaber* in a heterogeneously polluted environment. The hypothesis was that the tendency for aggregation outweighs the tendency for exploratory activity, therefore animals in groups will be less active. Consequently, this will affect their avoidance of polluted environmental patches. To test this hypothesis, isolated individuals or pairs of individuals were monitored in free-choice arenas where animals could select between uncontaminated and pyrethrin-contaminated soils. Animals were video recorded for 3 h in darkness using infrared light and analysed for avoidance behaviour and locomotor activity. In general, isolated animals were more locomotor active and avoided the contaminated soil more than paired animals. It can be concluded that aggregation behaviour suppresses exploratory behaviour and consequently also the avoidance of polluted environments. This should be accounted for when interpreting results of avoidance tests with groups of gregarious animals, which may underestimate the effect of pollutants.

Keywords

Aggregation, ecotoxicology, locomotion, Isopoda, Oniscidea, woodlice

Introduction

In the modern world not only heterogeneous distribution of resources but also environmental pollution is forcing animals to an exploratory behaviour. The shorter the time animals spend in a polluted environment, the more likely are they to survive. To do so, sensing, locomotor activity, spatial orientation, and appropriate storage of information about the new environment are crucial.

There is no doubt that terrestrial isopods can sense certain pollutants as many studies have shown their avoidance of soil or food polluted with metals (Zidar et al. 2003, 2005; Loureiro et al. 2005), veterinary pharmaceuticals (Žižek and Zidar 2013), pesticides (Loureiro et al. 2005; Santos et al. 2010; Zidar et al. 2012), char (Madžarić et al. 2018), nanoparticles (Zidar et al. 2019), or a mixture of pollutants (Loureiro et al. 2009). Terrestrial isopods have simple eyes that can perceive only light/dark contrasts, so these animals rather rely on their two pairs of chemosensory antennae for spatial orientation tasks such as finding food and suitable microhabitats, as well as to communicate with conspecifics and avoid predators (Schmalfuss 1998; Thiel 2010). As ancestrally aquatic animals, woodlice evolved many adaptations enabling their life in terrestrial habitats (Schmalfuss 1998; Hornung 2011; Richardson and Araujo 2015; Sfenthourakis et al. 2020). Most species are either endogean or epigean and nocturnal (Schmalfuss 1998). The latter are active during the night-time and spend the day sheltering in dark and damp places to avoid desiccation (Hassall and Tuck 2007; Tuf and Jerabkova 2008; Hornung 2011). Another behavioural adaptation to the terrestrial environment is gregariousness, i.e., a tendency to aggregate or associate with conspecifics (Hornung 2011; Broly et al. 2013). This behaviour significantly reduces water loss (Allee 1926; Broly et al. 2014), promotes body growth (Takeda 1984), increases reproductive performance (Souty-Grosset et al. 1990), and protects against predators (Broly et al. 2013). However, several studies have shown that at least in some woodlice species the tendency to aggregate can lead to sub-optimal outcomes (Devigne et al. 2011; Broly et al. 2012, 2015). This is because the attraction between conspecifics can outweigh individual preferences to other environmental stimuli. Loureiro et al. (2005) noticed that aggregation is also likely to affect woodlice avoidance to a polluted environment. The same was noticed by Zidar et al. (2012) in the study of woodlice behavioural response to insecticide pyrethrin.

Avoidance behaviour as an endpoint is frequently used in ecotoxicological studies to determine soil quality (ISO 2008, 2011; van Gestel et al. 2018). Avoidance tests are multiple choice experiments where a group of ten animals, usually earthworms, spring-tails, or isopods might select between two or more differently contaminated soils. The measured outcome of such tests is the number of animals on uncontaminated and contaminated soil after 48 h exposure. Advantages of this kind of avoidance tests are simplicity, short duration, and sensitivity that is comparable to acute or reproduction tests (Hund-Rinke and Wiechering 2001; Loureiro et al. 2005; Žižek and Zidar 2013; Madžarić et al. 2018; van Gestel et al. 2018). However, in gregarious animals like isopods, aggregation behaviour might seriously affect the results of avoidance tests.

In this study we evaluated the influence of gregariousness on the behavioural response of individuals in a heterogeneously polluted environment. We hypothesized

that the tendency for aggregation outweighs the tendency for exploratory activity, therefore animals in a group will be less active. Consequently, this will affect their avoidance of contaminated soil. To test this hypothesis, we monitored the locomotor activity and avoidance response of the terrestrial isopod *Porcellio scaber* Latreille, 1804 in a heterogeneously polluted environment.

Materials and methods

Experimental animals

Porcellio scaber is one of the most frequently used species in toxicity testing (van Gestel et al. 2018). The parent population of animals used in this study originated from an unpolluted environment near the city of Kamnik (46°12'01.8"N, 14°35'31.7"E) in Slovenia (Europe). Animals were bred for several years in large glass containers with a mixture of limestone sand and soil at the bottom, at room temperature (20–23 °C), high relative air humidity (~ 95%) and a natural diurnal light regime. They were left to feed ad libitum on maple (*Acer* sp.) leaf litter, with regular additions of carrots and potatoes.

For the experiments, laboratory raised individuals were used. On the day of the recording, adult male specimens in the intermoult phase (Zidar et al. 1998) were selected from the breeding containers. Body size (measured from the anterior edge of the head to the base of the uropods) of the selected isopods ranged between 10 and 12 mm.

Experimental set-up

Isolated individuals or individuals accompanied by a conspecific were monitored in free-choice experiments where animals could select between uncontaminated and pyrethrin-contaminated soils. For this purpose, circular transparent polypropylene (PP) pots meant for food packaging (diameter 9.5 cm, height 6.0 cm) were used as test arenas. Arenas were divided into two equally sized chambers with a 3.5 cm high PP barrier (Fig. 1). In the middle of the barrier was a passage (diameter of 1.2 cm), which was large enough for the isopods to easily pass through. At the bottom of the arena there was a 10 mm thick plaster of Paris darkened with charcoal (Sørensen et al. 1997).

Approximately 20 min prior recording a 3.0 g of Lufa 2.2 soil (Speyer, Germany) was added on top of the plaster that was previously saturated with tap water. Soil was previously dried, grinded, and sifted through a 0.5 mm sieve. Homogeneously granulated substrate prevented additional tactile stimuli that can affect animals' activity (Anselme 2013). In control groups uncontaminated soil was added to both test arena chambers simultaneously. In treatment groups uncontaminated and contaminated soils were added separately to each chamber in the following manner. First, the passage between the chambers was closed with an adhesive tape. Next, uncontaminated soil was added to one of the chambers (chamber A), previously marked with a number (Fig. 1). Afterwards, contaminated soil was added to the adjacent chamber (chamber B) and, finally, the adhesive tape blocking the passage was removed.



Figure 1. The two-chambered test arena for video tracking experiments with *Porcellio scaber*. Animals could select between uncontaminated (chamber A) and pyrethrin-contaminated soils (chamber B).

For each new recording a new substrate was prepared, both plaster and soil, but pots were re-used after thorough washing with tap water. This prevented any other chemical stimuli besides insecticides to affect animals' activity. For example, it is known that pheromones in woodlice faeces promote aggregation behaviour (Takeda 1980).

Soil contamination

Pyrethrins were used as a soil contaminant in all experiments in this study. Pyrethrins are chemicals of natural origin with insecticidal action that have been used to control pests indoor and outdoor since the early 1800's (Casida 1980; Tod et al. 2003). They prolong the openness of sodium channels in nerve cells, which paralyses the animal at higher doses. Therefore, longer exposure to an insecticide reduces animal's chances of survival.

Soil was contaminated for 1 h prior the recording with the insecticide product Flora Kenyatox Verde Plus (Unichem, Slovenia) which contained 0.2% of pyrethrin. The insecticide product was well shaken and 50 μ L, 100 μ L, 150 μ L, and 200 μ L was added to 20 g of dry soil and mixed well with a spatula. The obtained concentrations were 2.5, 5.0, 7.5, and 10.0 mL of insecticide formulation per kg dry soil which roughly correspond to 5.2, 10.4, 15.7, and 20.8 mg of pyrethrin per kg dry soil.

Video recording and observation of animals

One-hour prior video recording animals were marked dorsally across several tergites with partly dried enamel white paint to increase the visual contrast between woodlice and the substrate. In the case of two animals per arena only one animal was marked. It was reported previously that some external markers (e.g., nail polish) may affect diurnal activity and food consumption in some isopod species (Drahokoupilová and Tuf 2012; Kenne et al. 2019). However, our previous experiments have shown that the

marking we used is less persistent and has no effect on animal activity. Until recordings, animals were kept on moist plaster to prevent desiccation.

At the start of recordings one or two animals were placed in test arenas, always in chamber A containing uncontaminated soil. The animals were then continuously recorded for 3 h. In similar studies, animals' activity was tracked for 2 or 4 hours (Bayley et al. 1997; Sørensen et al. 1997; Engenheiro et al. 2005). Our preliminary observations have shown that *P. scaber* is highly active up to 3 h after introducing into the arena but then its activity decreases due to habituation (Anselme 2013), therefore recording longer than 3 h is unnecessary.

To provide a recording environment isolated from the outside light and other unwanted perturbances, a specially designed recording box was used. The box measured 55, 100, and 100 cm in depth, width, and height, respectively. Animals were recorded only under infrared light (850 nm) to avoid any light-induced behaviour (Devigne et al. 2011; Broly et al. 2015). Videos were captured with two webcams (Logitech C920) simultaneously. Cameras have been modified to improve video quality for recordings in infrared light. Each camera recorded two arenas. A 140-led IR illuminator was used to ensure adequate illumination of test arenas.

Videos were captured in VirtualDub 1.10.4 at 5 frames per second and a Full HD resolution (1920 × 1080 pixels). One pixel corresponded to 0.13 mm.

Video analysis

Videos were first analysed via video-tracking in Bonsai 2.4.0 (Lopes et al. 2015). The area of each test arena chamber was isolated by cropping. The white spot at the back of the marked animal was extracted from the background by thresholding and the spot's centroid coordinates inside each chamber were determined for each video frame. In the case of paired animals only the marked animal was analysed. Altogether 44 isolated and 44 paired animals were analysed ($n = 12$ for 0 mg of pyrethrin per kg dry soil; $n = 8$ for 2.5, 5.0, 7.5, and 10.0 mg of pyrethrin per kg dry soil).

Next, videos were examined also manually. In this way a sequence of several hundred frames when animals were at rest with antennae close to the body and no detectable movements was selected. Based on allocations of the centroid between two consecutive frames in this sequence the upper limit for noise was determined (0.2 pixels or 0.022 mm per frame). The upper limit for non-locomotor activity (0.8 pixels or 0.088 mm per frame) was determined based on a sequence of several hundred frames when animals were feeding or digging. All larger allocations of the centroid between two consecutive frames (> 0.8 pixels) were considered as locomotor activity. Additionally, the number of visits to contaminated soil (chamber B) was counted.

Finally, raw trajectories were imported to MS Excel and used to calculate behavioural variables: the proportion of time that isopods spent on the uncontaminated soil (chamber A), the overall duration of locomotor and non-locomotor activity. Change in animal's position during locomotor activity was calculated as path length. Average speed was calculated as the total path length divided by the total time of locomotor activity.

Data analysis

All statistical analyses were performed in R 4.1.1. (R Core Team), except for the probit regression and Pearson's correlation which were performed in SPSS 27.0. All plots were drawn using the latter software as well.

Sample sizes were relatively small, data were often non-normally distributed, and between-group variance was often heteroscedastic, therefore robust statistical methods implemented in the R package WRS2 were employed (Mair and Wilcox 2020). Instead of mean or median, a robust measure of central tendency was used, i.e., either a modified one-step *M*-estimator based on Huber's Psi (est parameter set to "mom") or a 20% trimmed mean (see below for details).

The avoidance response was determined in four different ways. In the first approach, we assumed that avoidance of contaminated soil was successful if animals spent more than half of the recording time on uncontaminated soil. Therefore, the percentage of time on uncontaminated soil was tested against a fixed value of 50% for each concentration and the control. To do so a robust one-sample test was applied using the function *onesampb()*, the *M*-estimator of central tendency, and 10,000 bootstraps (to estimate the 95% confidence intervals).

In the second approach, data on the time spent on uncontaminated soil were used to estimate the median effective concentration of pyrethrin (EC_{50}) for avoidance response. Data were first transformed by a formula adapted from ISO (2008): $AR = ((t_i - t_c) / t) * 100$ (t_i – time on contaminated soil; t_c – time on uncontaminated soil; t – total time of observation), and then the probit regression was applied. Negative values were considered as 0% of avoidance.

In the third approach, the avoidance response was assessed by the number of visits to contaminated soil. A robust two-way ANOVA was applied using the number of visits to contaminated soil as a dependent variable, while concentration treatment (0, 2.5, 5.0, 7.5, 10.0) and number of animals (isolated, paired) were used as independent categorical variables whose interaction was tested as well. For two-way ANOVA the function *pbad2way()*, the *M*-estimator of central tendency, and 5,000 bootstraps were used. Next, post-hoc comparisons were performed to find at which concentrations the response differed from the control in isolated (4 tests) and paired (4 tests) animals, as well as to find at which concentration treatments the response of isolated and paired animals was mutually different (5 tests). For this, robust independent two-sample tests were applied using the function *pb2gen()*, the *M*-estimator of central tendency, and 10,000 bootstraps (to estimate the 95% confidence intervals). *P*-values were adjusted via the method of Benjamini and Hochberg (1995).

In the fourth approach to estimate avoidance response, the location of animals at the end of the 3-h recordings was used. The percentage of animals on the uncontaminated soil was calculated.

Behavioural variables on isopod activity (duration, path length, average speed) were analysed as described for the third approach to avoidance response estimation. First, a robust two-way ANOVA was performed with the specific behavioural variable as a dependent variable, while concentration treatment and number of animals were used as independent categorical variables whose interaction was also tested. Upon significant effects, the

same post-hoc comparisons procedure as stated above was applied. Additionally, Pearson's correlation between the duration of locomotor activity and path length was calculated.

Data on average speed at contaminated (chamber B) and uncontaminated (chamber A) soil involved two measurements per individual. To account for repeated measurements, a robust two-way between-within subjects ANOVA was applied. Separate models were fitted for isolated and paired animals. In both, average speed was used as a dependent variable, while concentration treatment and arena chamber (A – uncontaminated, B – contaminated) were used as independent categorical variables whose interaction was also tested. In this case the function *butrim()* and the 20% trimmed mean as an estimate of central tendency were used. Next, post-hoc comparisons were performed to find at which concentration treatments average speed differed between the two arena chambers in isolated (5 tests) and paired (5 tests) animals. For this, robust dependent two-sample tests were applied using the function *yuend()* and the 20% trimmed mean as an estimate of central tendency. *P*-values were adjusted according to Benjamini and Hochberg (1995). Additionally, effect sizes, i.e., magnitude of between group mean differences, were calculated as proposed by Wilcox and Tian (2011).

Results

Avoidance response

Time spent on uncontaminated soil

Isolated animals of the control group spent, on average, the same amount of time in both chambers containing uncontaminated soil (Suppl. material 1: Table S1, Fig. 2). This was the case also in the control group of paired animals although some preference for chamber B was noticed.

Isolated animals showed avoidance behaviour to 5.0, 7.5, and 10.0 mL of pyrethrin formulation per kg dry soil as they spent significantly more time on uncontaminated soil (Suppl. material 1: Table S1, Fig. 2). In contrast, paired animals spent significantly more time on uncontaminated soil only at the highest concentration used, i.e., 10.0 mL/kg dry soil.

EC_{50}

The estimated EC_{50} for isolated animals was 2.8 mL of pyrethrin formulation per kg dry soil (95% confidence interval: 2.7–5.0 mL/kg) while EC_{50} for paired animals was 7.9 mL of pyrethrin formulation per kg dry soil (95% CI: 5.1–21.4 mL/kg), much higher than for isolated animals.

Number of visits to contaminated soil

The ANOVA showed that the number of visits to contaminated soil differed significantly between concentration treatments ($p = 0.01$) and number of animals ($p = 0.006$).

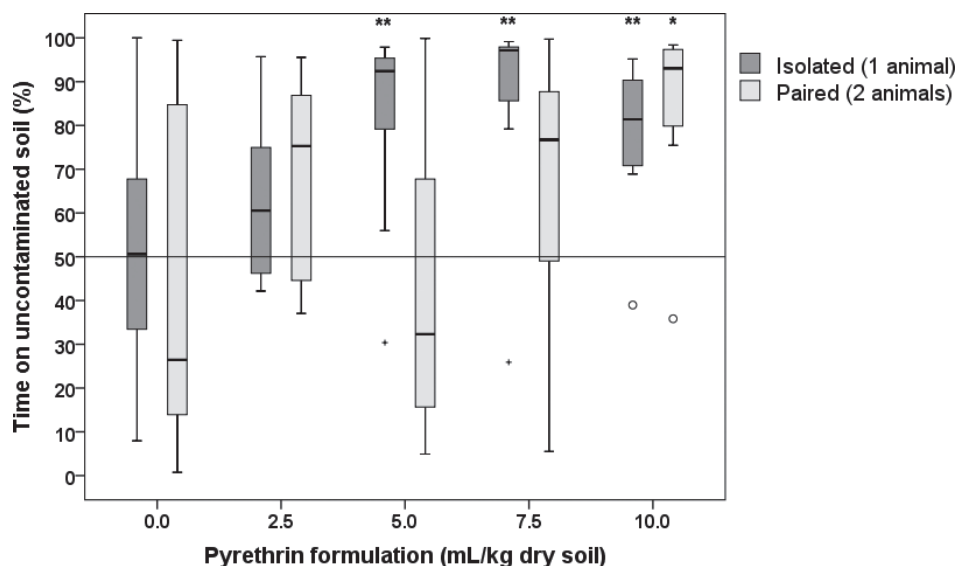


Figure 2. The percentage of time that *Porcellio scaber* spent on uncontaminated soil (chamber A) within the 3 h of observation. In a free-choice experiment isolated or paired animal could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} < 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; * – significantly different than 50%, $p < 0.05$; ** – as previous, but $p < 0.01$.

However, we found no interaction effect between these two variables ($p = 0.189$), meaning that the difference between isolated and paired animals did not differ among concentration treatments.

Post-hoc comparisons between different pyrethrin concentrations and the control revealed that in both isolated and paired animals the number of visits to contaminated soil significantly decreased at concentrations of 5.0 mL of pyrethrin formulation per kg dry soil or higher (Suppl. material 1: Table S2, Fig. 3). Moreover, post-hoc comparisons between isolated and paired animals at the same concentration treatment showed that paired animals visited contaminated soil less frequently compared to isolated animals in the control as well as at 5.0 and 10.0 mL/kg dry soil (Suppl. material 1: Table S2, Fig. 3). The same trend was observed also at 7.5 mL/kg dry soil, but the difference was only marginally statistically significant.

Location of animals after 3 hours

After 3 h, six isolated and five paired animals of control groups were found in chamber A, and the rest in chamber B. The location of pyrethrin exposed animals did not correspond to the concentrations of pyrethrin in soil (Table 1), or to the time spent on uncontaminated soil (Fig. 2). The highest avoidance in isolated animals was recorded at 5.0 mL/kg dry soil, while no avoidance was noticed at 10.0 mL/kg. In paired animals the highest avoidance was recorded at 7.5 mL/kg dry soil.

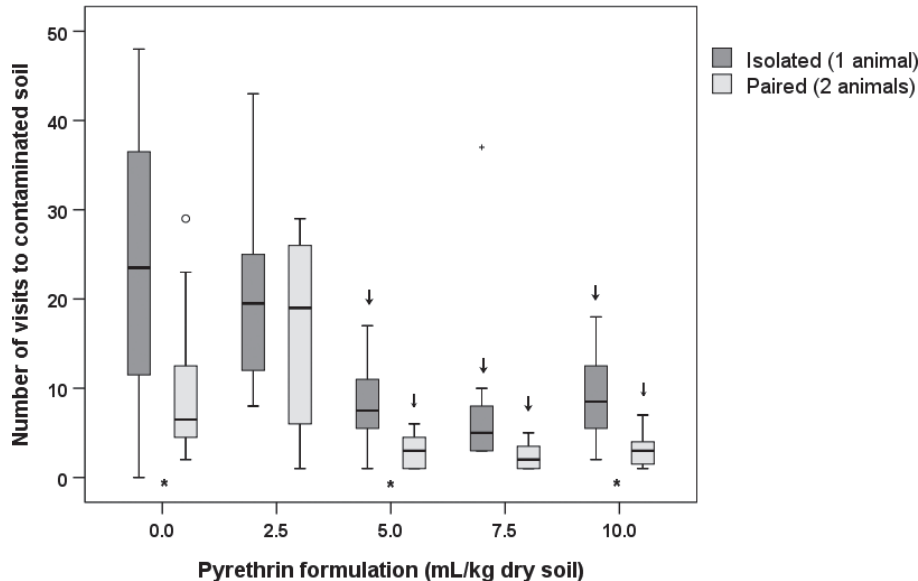


Figure 3. The number of visits to contaminated soil (chamber B) that *Porcellio scaber* made during the 3 h of observation. In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} > 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; ↓ – significantly lower than control, $p < 0.05$; * – significant difference between isolated and paired animals, $p < 0.05$; ** – as previous, but $p < 0.01$.

Table 1. The percentage of animals (*Porcellio scaber*) located on uncontaminated soil (chamber A) at the end of 3 h observation. In a free-choice experiment, animals could select between soil contaminated with pyrethrin and uncontaminated soil.

Pyrethrin concentration (mL/kg dry soil)	Isolated animals (%)	Paired animals (%)
0	50	42
2.5	50	62.5
5.0	92	25
7.5	75	87.5
10.0	50	75

Activity of animals

Locomotor activity

Duration of locomotor activity

In general, the animals were locomotory active from several minutes up to 1 h (Fig. 4). An exception were the control isolated animals, where more than half of the animals were locomotory active more than 1 h, some animals even more than 2 h. The ANOVA showed that the duration of locomotor activity differed significantly between concentration treatments ($p = 0.019$) and the number of animals ($p = 0.035$), but

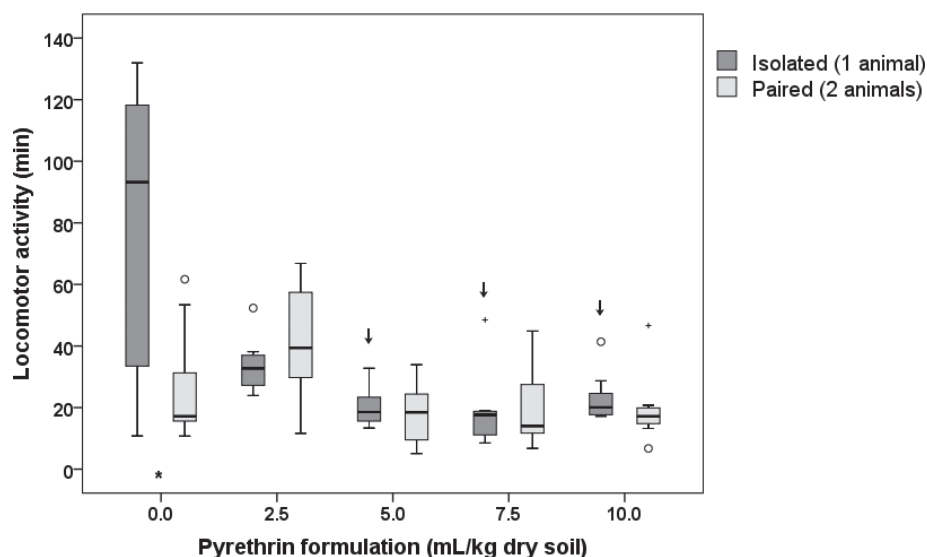


Figure 4. Duration of locomotor activity of *Porcellio scaber* within the 3 h of observation. In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} < 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; ↓ – significantly lower than control, $p < 0.05$; * – significant difference between isolated and paired animals, $p < 0.05$.

we found no interaction effect between these two variables ($p = 0.165$). The latter meaning that the difference between isolated and paired animals did not differ among concentration treatments.

Post-hoc comparisons between different pyrethrin concentrations and the control revealed that in isolated animals, locomotor activity decreased when exposed to pyrethrin formulation in soil (Suppl. material 1: Table S3, Fig. 4). The trend was statistically significant at all concentrations but 2.5 mL/kg dry soil. This was not the case with paired animals, which exhibited similar locomotor activity in control and treatment groups, and were most locomotory active when exposed to 2.5 mL of pyrethrin formulation per kg dry soil. Moreover, post-hoc comparisons between isolated and paired animals at the same concentration treatment showed significantly higher locomotor activity than paired animals (Suppl. material 1: Table S3, Fig. 4). However, when exposed to pyrethrin, the locomotor activity duration did not differ between isolated and paired animals.

Path length

The length of the path that the animals walked during the observation correlated with the duration of locomotor activity (Pearson correlation: $r = 0.893$, $p < 0.001$) and varied from 0.8 m up to nearly 37 m (Fig. 5). The ANOVA showed that the path length differed significantly between concentration treatments ($p = 0.009$) and number of animals ($p = 0.01$). However, no interaction effect was found between these two vari-

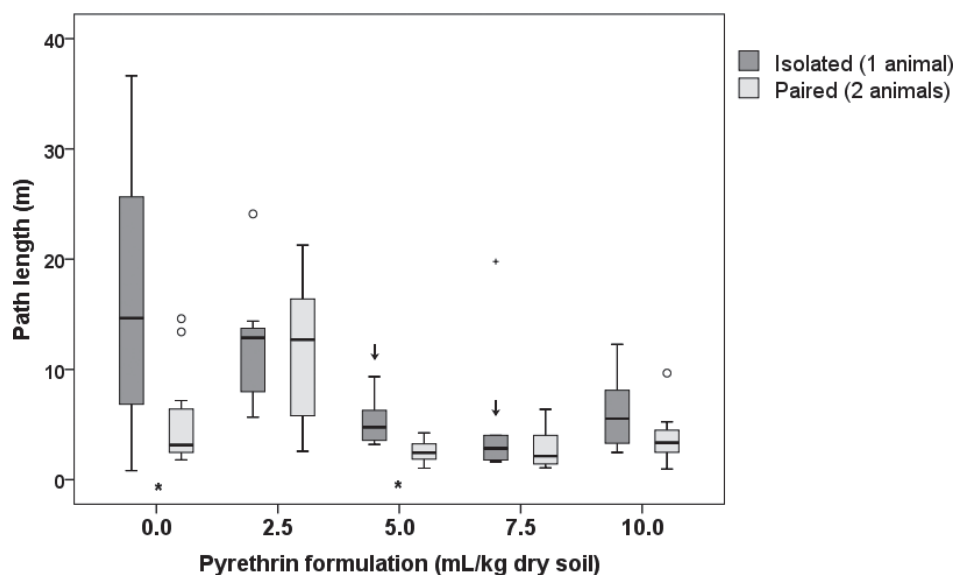


Figure 5. Overall length of the path that *Porcellio scaber* walked within the 3 h of observation. In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} < 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; ↓ – significantly lower than control, $p < 0.05$; * – significant difference between isolated and paired animals, $p < 0.05$.

ables ($p = 0.168$), meaning that the difference between isolated and paired animals did not differ among concentration treatments.

Post-hoc comparisons between different pyrethrin concentrations and the control, revealed that in isolated animals the path length decreased with increasing concentration of pyrethrin formulation in soil, but was significantly lower than the control only at concentrations of 5.0 and 7.5 mL/kg dry soil (Suppl. material 1: Table S4, Fig. 5). In paired animals exposed to pyrethrin, the path length did not differ from the control at any concentration, but animals exposed to 2.5 mL/kg dry soil made the longest path. Furthermore, post-hoc comparisons between isolated and paired animals at the same concentration treatment showed no clear pattern in their mutual differences (Suppl. material 1: Table S4, Fig. 5). However, at the control and 5.0 mL/kg dry soil, the path length was significantly higher in isolated animals compared to paired animals.

Average speed

The average speed of locomotion of control animals was 1.3–4.6 mm/s in isolated animals and 2.2–4.2 mm/s in paired animals (Fig. 6). The ANOVA showed that average speed differed significantly between concentration treatments ($p = 0.002$) and number of animals ($p = 0.003$), but no interaction effect between these two variables was found ($p = 0.238$). The latter meaning that the difference between isolated and paired animals did not differ among concentration treatments.

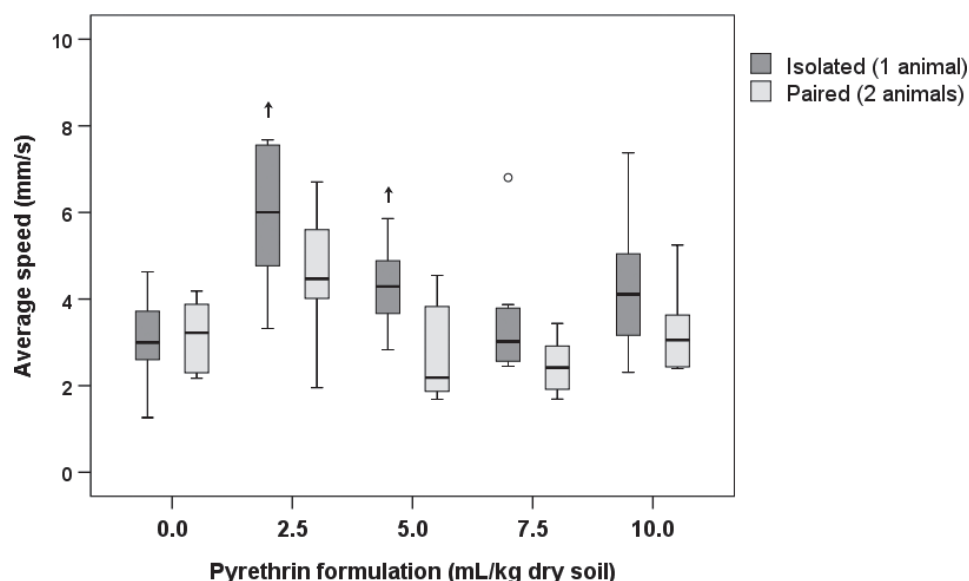


Figure 6. Average speed of locomotion of *Porcellio scaber* within the 3 h of observation. In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: 3 IQR \leq value > 1.5 IQR; + – extreme: value > 3 IQR; \uparrow – significantly higher than control, $p < 0.05$.

Post-hoc comparisons between different pyrethrin concentrations and the control revealed that in isolated animals the average speed significantly increased at 2.5 and 5.0 mL/kg dry soil (Suppl. material 1: Table S5, Fig. 6). In pyrethrin exposed paired animals, the average speed was not significantly different from the control at any concentration. In addition, post-hoc comparisons between isolated and paired animals at the same concentration treatment showed non-significant differences in average speed in all cases (Suppl. material 1: Table S5, Fig. 6). However, the general pattern of higher average speeds in isolated compared to paired animals exposed to pyrethrin was supported by the significant ANOVA result (see above).

Further analyses focused on average speed of isolated and paired animals in the two arena chambers (uncontaminated vs. contaminated) at different pyrethrin concentrations. The ANOVA for isolated animals showed that their average speed depended on the arena chamber ($p = 0.001$) and concentration ($p = 0.015$), but the interaction of these two variables had no significant effect on the response ($p = 0.268$). The latter meaning that the difference in average speed between uncontaminated and contaminated soil did not differ among concentration treatments. Post-hoc comparisons revealed that isolated animals moved significantly faster on contaminated soil than on uncontaminated soil at 2.5 and 10.0 mL of pyrethrin formulation per kg dry soil (Suppl. material 1: Table S6, Fig. 7A). The same trend was observed at 5.0 and 7.5 mL/kg dry soil, but the differences were only marginally significant. However, note that the effect sizes were large also for the latter two comparisons.

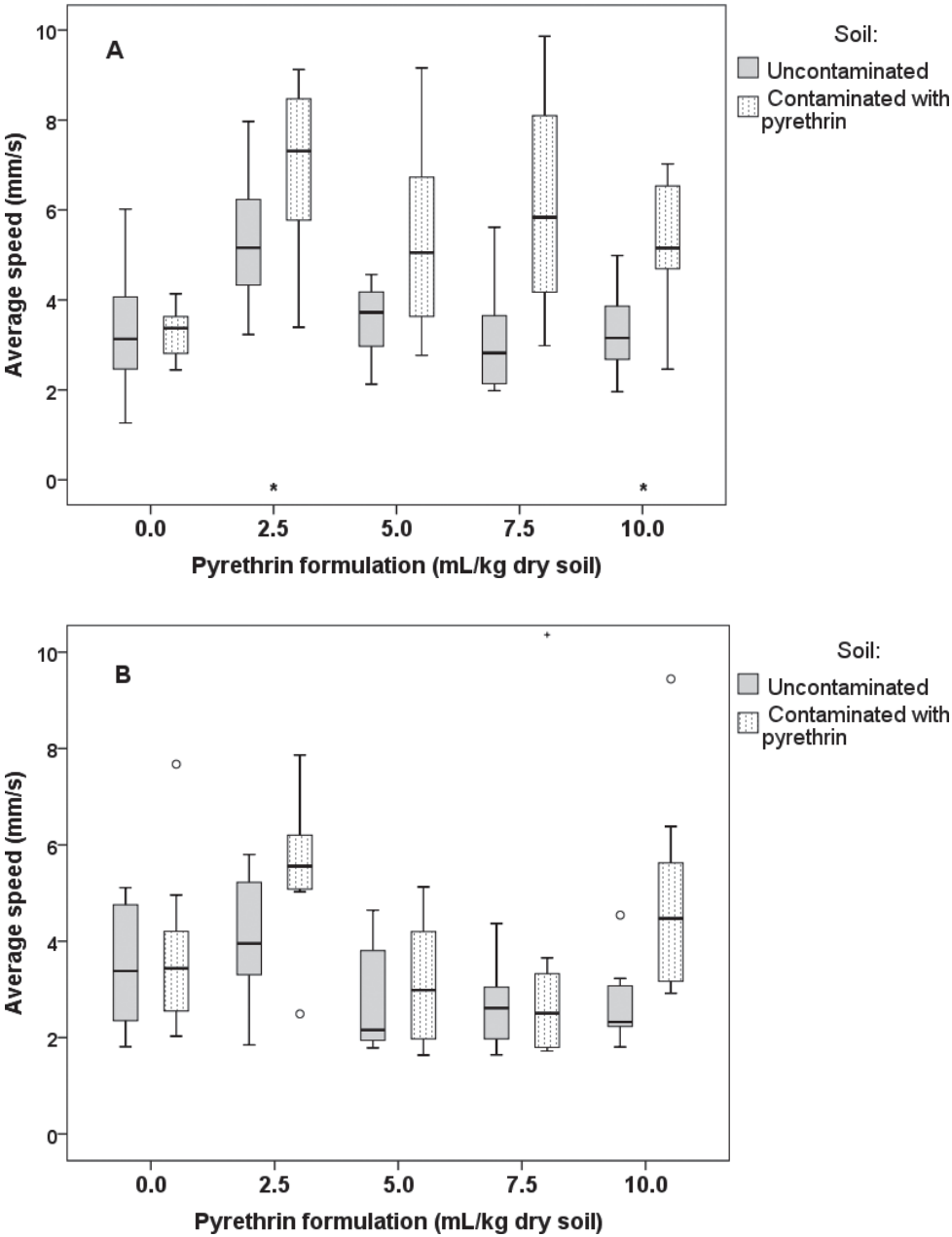


Figure 7. Average speed of locomotion of *Porcellio scaber* on uncontaminated soil (chamber A) and soil contaminated with pyrethrin (chamber B) for isolated animals (A) and paired animals (B). In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. In plot A, two extreme values for chamber B are not shown: at conc. 2.5 (value = 12.99) and conc. 10 (value = 25.77). Key: box: 25th, 50th, and 75th percentile; whiskers: value \leq 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} < 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; * – significant difference between uncontaminated and contaminated soil, $p < 0.05$.

The ANOVA for paired animals showed that their average speed depended on the arena chamber ($p = 0.003$) and concentration ($p = 0.007$), as well as the interaction of these two variables ($p = 0.045$). The significant interaction effect reveals that the difference in average speed between uncontaminated and contaminated soil differed among concentration treatments. Post-hoc comparisons revealed that paired animals did not move significantly faster on contaminated soil compared to uncontaminated soil at any concentration treatment, although marginally significant differences and large effect sizes in this direction were observed at concentrations 2.5 and 10.0 mL/kg dry soil (Suppl. material 1: Table S6, Fig. 7B). These are most likely also the reason for the significant interaction effect observed in ANOVA (see above).

Non-locomotor activity

Non-locomotor activity of animals lasted from 8 to 49 min (Fig. 8). The ANOVA showed that non-locomotor activity differed significantly between concentration treatments ($p = 0.015$), but not between the number of animals ($p = 0.797$). A significant interaction effect between these two variables ($p < 0.001$) was also observed, meaning that the difference between isolated and paired animals in non-locomotor activity differed among concentration treatments.

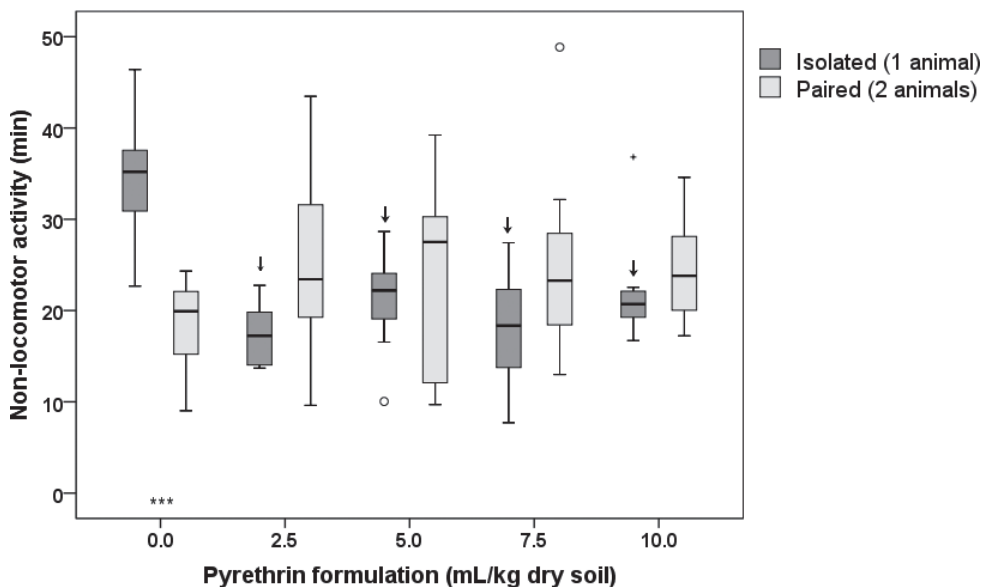


Figure 8. Duration of non-locomotor activity of *Porcellio scaber* within the 3 h of observation. In a free-choice experiment, isolated or paired animals could select between soil contaminated with pyrethrin and uncontaminated soil. Key: box: 25th, 50th, and 75th percentile; whiskers: value ≤ 1.5 IQR; o – outlier: $3 \text{ IQR} \leq \text{value} < 1.5 \text{ IQR}$; + – extreme: value $> 3 \text{ IQR}$; ↓ – significantly lower than control, $p < 0.01$; *** – significant difference between isolated and paired animals, $p < 0.001$.

Post-hoc comparisons between different pyrethrin concentrations and the control revealed that in isolated animals, non-locomotor activity significantly decreased at all pyrethrin concentrations used (Suppl. material 1: Table S7, Fig. 8). This was not the case in paired animals for which no significant differences between the control and pyrethrin concentrations were observed. Post-hoc comparisons between isolated and paired animals at the same concentration treatment showed that in control the duration of non-locomotor activity was significantly higher in isolated animals (Suppl. material 1: Table S7, Fig. 8). When animals were exposed to soil contaminated with pyrethrin, non-locomotor activity tended to be higher in paired animals, but the differences were statistically non-significant at all concentrations. This pattern is most likely also the reason for the significant interaction effect observed in ANOVA (see above).

Discussion

We investigated the influence of aggregation on avoidance behaviour and activity of *Porcellio scaber* exposed to contaminated soil. Individual animals (isolated) or animals in pairs were recorded for 3 h in two chambered arenas where they could select between uncontaminated soil and soil contaminated with the insecticide pyrethrin. Time spent on uncontaminated soil revealed more successful avoidance of contaminated soil in isolated than in paired animals. This measure of avoidance response was more sensitive and robust than the number of visits to contaminated soil or location of animals after a specific time since exposure. Animals unexposed to contaminated soil were significantly more active when isolated than when in pairs. This was evident from the duration of locomotory and non-locomotory activity. However, when exposed to pyrethrin the differences between isolated and paired animals decreased, although some differences in path length and average speed still indicated higher activity of isolated animals.

Avoidance response

Although all animals started the experiment in chamber A, the control animals showed that the initial position of animals in the arena does not affect the time the control animals spent in each chamber of the arena (A or B, both containing uncontaminated soil). In published avoidance behaviour test protocols, animals were introduced into the test arenas differently: in the middle between control and test soils (Loureiro et al. 2005), randomly on both soils (Zidar et al. 2012; Škarkova et al. 2016) or on the uncontaminated soil (Zidar et al. 2019; the present study). Our result confirmed previous reports by Anselme (2013, 2015), that *P. scaber* shows high exploratory activity in a new environment and can explore both chambers of the arena in a short period of time.

During 3 h of exposure isolated isopods clearly avoided soil with pyrethrin formulation already at a concentration of 5.0 mL of formulation per kg dry soil, which roughly corresponds to 10.4 mg of pyrethrin per kg dry soil. This concentration is the lowest observed effective concentration (LOEC) in this study. According to our previ-

ous study (Zidar et al. 2012), this concentration can be considered as an upper sublethal concentration for *P. scaber*. In paired animals, the LOEC was twice as high, i.e., 20.8 mg/kg dry soil, at the concentration that is considered lethal to *P. scaber* (Zidar et al. 2012). Such weaker or delayed avoidance response in paired animals effectively means longer exposure to pyrethrin. Potentially, locomotor dysfunctions that the insecticide may induce makes the isopods even harder to retreat from the contaminated soil. Such positively reinforced feedback loop due to insecticide poisoning was reported previously for springtails exposed to dimethoate (Pereira et al. 2013).

The EC_{50} value obtained in this study for isolated animals (2.8 mL of formulation or 5.9 mg of pyrethrin per kg dry soil) was almost twice lower than the EC_{50} value obtained in the 48-h avoidance test with animals in groups (9.7 mg of pyrethrin per kg dry soil; Zidar et al. 2012). This was so even though in the latter study pyrethrin formulation contained piperonyl butoxide that enhances the effects of pyrethrin (Kakko et al. 2000). In paired animals the obtained EC_{50} (7.9 mL of formulation or 16.5 mg of pyrethrin per kg dry soil) was almost 3× higher compared to isolated animals and also considerably higher than in our previous study (Zidar et al. 2012).

The frequency at which isolated animals visited contaminated soil decreased with the increased concentration of pyrethrin in soil and was in concordance with the time that animals spent on the contaminated soil. In paired animals the frequency of visits to contaminated soil was generally lower than that of isolated animals but their retention time on contaminated soil was much higher. As reported by Zidar et al. (2003), the number of visits does not necessarily reflect the exposure time.

The location of animals (on uncontaminated vs. contaminated soil) at a given time also does not necessarily reflect the avoidance response as has been reported previously (Odendaal and Reinecke 1997; Pereira et al. 2013). In our study, animals have often passed between chambers and after 3 h many animals were observed on contaminated soil, although they spent significantly more time on uncontaminated soil. However, the discrepancy between the animals' location at a given time and avoidance success can be avoided by using average time spent on uncontaminated soil as a measure of avoidance (this study), or to record the location of animals during the exposure more frequently (see Madžarić et al. 2018).

Our results show that the accepted standard toxicity tests relying on avoidance behaviour of a group of individuals as an endpoint should probably be reconsidered when performed with gregarious animals like isopods that exhibit strong aggregation behaviour. These tests tend to underestimate the effect of the toxicant. The reason for this is twofold and originates from the dynamic hierarchy of the two independent stimuli of an opposite sign provided by the toxicant (negative) and the presence of conspecifics (positive) against the background environment (neutral). When isopods are introduced to a novel environment such as the test arena, they first explore it in approximately random movement. Note that the location of the negative stimuli is fixed while the positive stimuli move(s) randomly within the arena. When both stimuli appear on different halves of the arena the choice is clear. The dilemma arises when both negative and positive stimuli occur at the same arena half. In such scenarios, the animals' response will depend on the relative strength of both stimuli. When toxicant

concentration is high, it will prevail over conspecific attraction and animals will eventually aggregate at the optimal arena half. However, when toxicant concentration is low, conspecific attraction prevails over the negative effects of toxicant and animals will aggregate at the non-optimal arena half. The observed result is a lack of avoidance response interpreted as no effect of toxicant. However, when individual animals, and not groups, are tested at the same low toxicant concentration, they show clear avoidance signalling harmful effects. Thus, aggregation can mask the real effect of the toxicant and tests with groups of animals will tend to overestimate the effective toxicant concentration.

The real concern is that in natural populations animals will practically always be in a group. Groups of aggregating animals avoid high toxicant concentrations but are much less effective at avoiding low and moderate concentrations although harmful. Consequently, in a heterogeneously polluted environment these concentrations might eventually cause more damage as animals will be exposed to them longer and accumulate their negative effects, while they will retreat from higher concentrations. The standard toxicity tests with avoidance behaviour will however fail to reveal this. Thus, for gregarious animals we should rather estimate the effective concentration for both individual animals and those in a group. Although counterintuitive at first sight, the range of concentrations between these two values may be effectively most harmful to the natural populations.

Finally, group size and social composition of its members are additional factors that for sure add to the variation of toxicity tests results and should be considered in model species for which this is relevant and possible. Further investigation is needed in this direction. From the broadest perspective, aggregation of gregarious animals will likely affect the outcome and interpretation of any kind of choice tests with any kind of aversive (e.g., light, predator pheromones) or favourable (e.g., humidity, thigmotactic shelters) stimuli.

Activity of animals

Locomotor behaviour of terrestrial isopods was recognised as a sensitive biomarker of exposure to different pollutants (Bayley et al. 1995, 1997; Sørensen et al. 1997; Engenheiro et al. 2005). Bayley et al. (1995) reported increased locomotory time, speed, and path length of *P. scaber* when exposed to sublethal concentrations of insecticide dimethoate. Later, studies showed that increased locomotor activity strongly correlate with the assimilated dimethoate and consequently acetylcholinesterase inhibition (Bayley and Beatrupe 1996; Jensen et al. 1997). On the other hand, Engenheiro et al. (2005) reported a decreased locomotory activity and path length of *Porcellionides pruinosus* exposed to higher concentrations of dimethoate. Decreased locomotor activity was recorded also in *Oniscus asellus* collected at polluted sites (Bayley et al. 1997; Sørensen et al. 1997). In our study, in contrast to the above examples, the choice for uncontaminated soil was offered, the locomotor and non-locomotor response and path length of isolated animals to pyrethrin contaminated soil was reduced. The activity of paired animals was generally low, in control and exposed animals, therefore exposed animals did not differ from the control animals. The total activity in control

groups, non-locomotor and locomotor activity together, ranged 16–54 min/h in isolated and 7–27 min/h in paired animals. The activity of control isolated animals was comparable to control animals from the study of Bayley et al. (1995), while the activity of our paired animals was lower. The next obvious difference between isolated and paired isopods was the speed of locomotion on contaminated soil. Isolated animals were moving on contaminated soil much faster than on uncontaminated soil, but this was less obvious in paired animals. Faster locomotion in an environment with adverse stimuli is expected as it enhances the chance of finding a more suitable space, while slowing down can indicate the onset of favorable conditions (Fraenkel and Gunn 1961; Breed and Moore 2021). Although the rate of locomotion is often proportional to the intensity of the adverse stimulus, we did not observe such a response. On the other hand, different speed of locomotion on contaminated and uncontaminated soil also indicated that lower activity of exposed animals has not been caused by pyrethrin poisoning, as reported for dimethoate (Bayley and Beatrupe 1996).

Conclusions

We conclude that:

- lower activity of aggregated animals leads to a less successful avoidance of moderately contaminated soil;
- aversive stimuli of pollution force animals to move faster on contaminated soil if not suppressed by aggregation behaviour;
- attraction between individuals might outweigh aversive stimuli of pollution leading to longer exposure to pollutant;
- aggregation behaviour should be accounted for when interpreting results of avoidance tests with groups of gregarious animals, which may underestimate the effect of pollutant.

Acknowledgements

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Supplementary material I

Table S1–S7

Authors: Primož Zidar, Žiga Fišer

Data type: Statistical data

Explanation note: Avoidance behavior toxicity tests should account for animal gregariousness: a case study on isopod *Porcellio scaber*.

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Antipredatory strategies of terrestrial isopods

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Abstract

Terrestrial isopods (Oniscidea) represent a widespread group of land Crustacea that have been able to successfully adapt to the terrestrial environment and occupy newly formed ecological niches. During the colonisation of land, they faced numerous challenges, including finding an effective way to avoid their new terrestrial predators. In response to predation pressure, they have developed various behavioural and morphological adaptations. These include tonic immobility, conglobation, clinging to the ground, releasing strongly acidic secretions, jumping, and efficient running away. Furthermore, terrestrial isopods can aggregate with other individuals, use stridulation, or change their typical activity time. Some of them also developed spiny tergites and aposematic colouration or posture. The majority of these strategies have not yet been studied.

Keywords

Aggregation, aposematism, behavioural traits, death feigning, defence, mimicry, Oniscidea

Introduction

Oniscidea, commonly called terrestrial isopods, woodlice or pill bugs, represent one of the eleven suborders belonging to the order Isopoda (Pericarida, Crustacea) that first appeared on land during the lower Carboniferous (Schram 1982; Fu et al. 2010). According to Broly et al. (2013), the oldest fossils of Oniscidea come from the Early Cretaceous, but some indications suggest that they could have already appeared in the

pre-Pangaeon period, most likely in the Carboniferous interval of the Late Palaeozoic. They were able to colonise various terrestrial habitats ranging from sea level to high mountains (Hornung 2011), and are represented by ~ 4,000 species distributed in more than 500 genera and 38 families (Javidkar et al. 2015; Sfenthourakis and Taiti 2015; Dimitriou et al. 2019; WoRMS 2021). Thus, Oniscidea represents the most flourishing crustacean group that has ever colonised the land. As mentioned in many previous studies, oniscid isopods must evolve various adaptations for their terrestrial life. They had to solve ecological and physiological problems, such as respiration, feeding, locomotion, reproduction, and at the same time protect themselves against desiccation in their new terrestrial habitats (Hornung 2011).

As terrestrial isopods colonised land some 300 MA, they faced several predators, such as centipedes, spiders, amphibians, and reptiles. Predator pressures caused Oniscidea to develop various morphological and behavioural adaptations. The lifespan of terrestrial isopods ranges from 1 to ~ 10 years. The highest mortality rate is within the first month of their life outside the brood pouch; this is most frequently due to climate factors, such as high temperatures, drought, and floods, as well as due to cannibalism of different ontogenetic stages (Brereton 1957). As previously mentioned, terrestrial isopods are the prey of a wide variety of predators. Additionally, for some of them (e.g., small passerines such as flycatchers or wrens), terrestrial isopods are a main source of calcium (Bureš 1986; Krištín 1992). Also, there are specialists, such as *Scorpio maurus palmatus* (Ehrenberg, 1828), whose major prey consists of the desert isopod *Hemilepistus reaumurii* (Milne Edwards, 1840) at some localities (Warburg 1993). Other well-known specialists are spiders of the genus *Dysdera* (Řezáč and Pekár 2007), with numerous species using at least three different hunting strategies by means of their specifically adapted chelicerae.

Predators keep up with antipredator adaptations of prey and improve their own hunting techniques accordingly. This never-ending struggle causes the creation of numerous adaptations of both predators and prey, i.e., terrestrial isopods. Although the various types of antipredation strategies among animals are well known, a comprehensive overview on the subject concerning terrestrial isopods is lacking. Therefore, this text provides a systematic review of currently known information regarding the antipredation strategies of terrestrial isopods. The known methods of terrestrial isopod defence against predators, both behavioural as well as morphological, are summarised below, including tentatively proposed strategies.

Not to be there

The simplest strategy to defend oneself against a predator is to keep one's distance from it, and to avoid an encounter with it. A useful strategy is to avoid staying in places where other individuals were killed. Supporting a necromone hypothesis, Yao et al. (2009) experimentally confirmed that terrestrial isopods are deterred by chemical substances (mainly linoleic acid) released by smashed terrestrial isopods (mainly their conspecifics).

In general, movement in a linear path represents the most efficient adaptive escape strategy when precise information about environmental risks for an animal is lacking (Jander 1975). In an open space, terrestrial isopods like *Armadillidium vulgare* (Latreille, 1804) move forward in a straight line (Iwata and Watanabe 1957). When *A. vulgare* and *Porcellio laevis* (Latreille, 1804) were kept in a box with the ants *Tetramorium caespitum* (Linnaeus, 1758), both species escaped the shelters and were more active outside of them as a result of ant harassment. The same effect was caused by an indirect cue from the predatory ants: both species of isopod kept themselves further away from the source of the ant odour (Castillo and Kight 2005).

As terrestrial isopods usually live on the soil surface, it is difficult for them to walk straight and maintain their direction while walking around numerous obstacles (e.g., stones, pebbles, and vegetation stems). The most effective way for them to keep a straight-forward direction is through systematic turn alternations (Hughes 1967). This is the way to reach the greatest distance from a starting point with the highest level of probability (Hughes 1989; Moriyama and Migita 2004). This behavioural pattern is shared by a diverse range of animals, including humans (Lepley and Rice 1952; Grosslight and Harrison 1961; Pate and Bell 1971).

A sophisticated method for testing the stress of terrestrial isopods is by keeping track of their movements through a T-maze (Ono and Takagi 2006). Terrestrial isopods use turn alternation as their strategy to escape from unpleasant places, and the intensity of the turn alternation is used to evaluate their level of stress (Houghtaling and Kight 2006). *Porcellio scaber* Latreille, 1804 more intensively alternated turns when they met a spider (e.g., the dangerous *Dysdera crocata* CL Koch, 1838) than when they met a harmless fly or cotton flock (Carbines et al. 1992). Chronic stress induced by indirect predatory cues (e.g., ant odour) can lead to increased turn alternation in *P. laevis* and *A. vulgare* (Hegarty and Kight 2014), behaviour that leads to the desertion of a dangerous place. Also, these isopods can use small chronical substrate vibrations for the detection of a predator (Zimmerman and Kight 2016). Habituation to the disturbance can significantly reduce turn alternation (Houghtaling and Kight 2006). The similarity in turn alternation of distantly related species of terrestrial isopods suggests evolutionary conservation of these antipredator mechanisms (Hegarty and Kight 2014). It is also known that this escape behaviour is a result of the isopod's own decision-making (Moriyama 1999; Moriyama and Migita 2004) and that *A. vulgare* can correct its turns to increase its level of alternation (Moriyama et al. 2016).

Terrestrial isopods not only engage in spatial predator avoidance, but they also engage in temporal predator avoidance. Terrestrial isopods can avoid encounters with a predator by changing the time range in which they are active. For example, while most terrestrial isopods are nocturnal, the burrowing isopods *H. reaumurii*, from the arid regions of North Africa, the Middle East, and Central Asia, are active in the colder season during full daylight (Nasri-Ammar and Morgan 2005). During the warmest months when the temperature can increase to 45 °C, *H. reaumurii* becomes active before sunrise, thereby exposing themselves to *Scorpio maurus*, their main nocturnal predator. Therefore *H. reaumurii* can switch its typical terrestrial isopod activity from

night to day for the sake of reducing the rate of its predation by scorpions. Although no study confirming this claim has been published yet, similar behaviour has been observed in other animals. For example, European rabbits have switched from nocturnal to diurnal activity after the appearance of European polecats, a typical night-time predator, in an area where rabbits were already present (Bakker et al. 2005).

Not to be seen

The tendency “not to be seen” can be an escape mechanism from predators. Some terrestrial isopod species, the so-called “runners” group according to the ecomorphological classification of Schmalzfuss (1984), have well-developed eyes and a relatively narrow body with long pereopods; these traits make them suitable for a quick escape (Schmalzfuss 1984). For example, *Philoscia muscorum* (Scopoli, 1763) is well adapted for a fast and surprising retreat thanks to its slim body and long legs (Sutton 1972). An astonishing antipredatory strategy related to “not to be seen” is diving (Leistikow 2001). An example is *Ischioscia hirsuta* Leistikow, 2001, which can hide under the water surface of small streams if disturbed.

A jumping strategy is more unpredictable than a running strategy for a hunter. For several species of *Philoscia* (*Ischioscia*), jumping, akin to springtails (Collembola), was reported (Williams 1941). Van Name (1925, 1926) mentioned leaping or jumping for *Ischioscia nitida* (Miers, 1877, but considered as nomen dubium) and *Ischioscia variegata* (Dollfus, 1893). Leistikow (2001) compared the jumping distance of *I. variegata* and *Ischioscia pariae* Leistikow, 2001 with each other. He described how the first species was recorded jumping up to 20 cm, while the second was recorded only jumping ~ 5 cm. Such distances should be far enough to avoid hunters (e.g., a spider). Leistikow (2001) also mentioned that a 20 cm-long jump was sufficient to escape human collectors. Jumping terrestrial isopods were reported also from Borneo, a species of *Burmoniscus*, and were subsequently assigned to the ecomorphological type “jumper” (Cranbrook and Edwards 1994; Hassall et al. 2006); this, despite the fact that jumpers and runners differ only in behaviour.

Visually oriented larger predators, such as amphibians, lizards, or birds, are attracted by the movement of prey. A very simple strategy related to “to not be seen”, not only used by terrestrial isopods, is to stay inactive when disturbed. When *A. vulgare* and *P. laevis* detect a predatory spider, they reduce their activity as a response (Zimmerman and Kight 2016). This behavioural strategy is typical for species of the ecomorphological group “clingers” (Schmalzfuss 1984), which have short strong legs, and can cling firmly to the substratum. Their dorsal part is protected by a strong exoskeleton that has broad tergites that expand their body shape, making it impossible to catch them or to turn them when clinging onto the substrate. Another strategy which is, for instance, characteristic for species living on tree bark is to fall down. When a bird tries to detach a terrestrial isopod from a bark, there is a high probability that the isopod will fall off the tree and become lost in the leaf litter around the base of the tree trunk (cf. Butler

1889). The subsequent immobility causes invisibility by the cryptic colouration of the terrestrial isopods, stained by different shades of grey, brown, and beige (Achouri and Charfi-Cheikhrouha 2009).

The tendency of terrestrial isopods to not instigate predators by their movement can also be related to “tonic immobility”. This is the state of reversible physical immobility and muscle hypertonicity during which the animals do not respond to external stimuli (Gallup 1974). Immobility is an often-used form of passive anti-predator behaviour adopted by a wide scale of animals, including terrestrial isopods (Quadros et al. 2012; Tuf et al. 2015). Tonic immobility is not a simple synonym of death feigning, i.e., thanatosis. Thanatosis is not necessarily tonic, such as in invertebrates or opossums (Francq 1969); it can also be in a relaxed state, such in some birds, mammals, or snakes (Holmes 1916). Moreover, the typical posture of a tonically immobile individual is usually unlike the posture of a genuinely dead individual, as mentioned by Darwin (cf. Holmes 1916). Typical tonic immobility posture of the clinger ecomorphological group of terrestrial isopods was described by Quadros et al. (2012) as follows: “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 backward and pressed against the dorsal contour of the first pereonites”. Terrestrial isopods of the ecomorphological group “rollers” (Schmalfuss 1984) adopt specific ball-like postures, this behaviour is called conglobation (or volvation, cf. Verhoeff 1930).

While adopting a posture increases the protection of an animal against being swallowed by a predator (Tuf et al. 2015), feigning death reduces the probability of being seen by predators. Thus, tonic immobility is a defence strategy against visually oriented predators. There are also some indications that the duration of thanatosis depends on the daily light regime. This was recorded in the freshwater crab *Trichodactylus panoplus* (von Martens, 1869) (Zimmermann et al. 2009) and the coleopteran *Cylas formicarius* (Fabricius, 1798) (Miyatake 2001). Another factor that can influence responsiveness to tonic immobility is temperature (Miyatake et al. 2008). Additionally, the type of stimulus can influence responsiveness, as was proved by Quadros et al. (2012) in their study of three terrestrial isopods *Balloniscus glaber* Araujo & Zardo, 1995, *Balloniscus sellowii* (Brandt, 1833), and *Porcellio dilatatus* Brandt, 1833. The duration of tonic immobility varies intraspecifically, and is related to the survival probability of prey. The antipredator behaviour of terrestrial isopods can be age-dependent, and may change during their life course. For example, *B. sellowii* uses tonic immobility more often when young and small when compared with older and larger individuals that employ more active escape strategies, such as running (Quadros et al. 2012). Body size can also play a crucial role in the effectiveness of tonic immobility because smaller animals are more likely to be disregarded by a predator. The discrepancy between escape and tonic immobility, both effective strategies, can lead to distinguishable personalities of terrestrial isopods, as shown for *P. scaber* (Tuf et al. 2015). Thus, terrestrial isopods can increase their survivorship using tonic immobility in one of two ways: they can either increase their resemblance with the surrounding environment and be less visible (Bergey and Weis 2006), or they can protect their vulnerable ventral surface (Quadros et al. 2012).

Not to be bitten

The soft vulnerable ventral surface of any terrestrial isopod is best protected during conglobation, which allows them to survive in conditions that may be lethal to other species (White and Zar 1968). This behaviour can be found among mammals, such as pangolins, hedgehogs, echidnas (Sigwart et al. 2019), tenrecs, and armadillos. It is also typical for arthropods such as pill millipedes, giant pill millipedes, soil mites, cuckoo wasps (Tuf et al. 2015), multi-shelled chitons (Eernisse et al. 2007), beetles (Ballerio and Grebennikov 2016), cockroaches (Perry and Nalepa 2003), trilobites, and some larvae of other groups (Haug and Haug 2014). This tonically immobile posture is typical among members of the families Armadillidae, Armadillidiidae, Cylisticidae, Tylidae, Helleriidae, Buddelundiellidae, Scleropactidae, or Eubelidae.

The ability to conglobate depends on several body characteristics. The bend of the tergites and the ventral muscles are the most important features that enable conglobation. Aside from an animal's arched shape, there are a wide range of additional body part adaptations common for species capable of conglobation. These include the shape of the head, the shape and length of the antennae, the shape of the epimers of the pereonites, and the shape of the pleon, telson, and uropods. Species with the ability to roll up have often developed head grooves in which the antennae can fit (Sutton 1972). Also, conglobation leads to some adaptations of the female's marsupium (a brood pouch in peracarid crustaceans). In ovigerous females, the oostegites allow them to bend enough to conglobate (Csonka et al. 2015). As consequence of conglobation, the female's internal organs are compressed and displaced, with the eggs are condensed into the anterior part of the marsupium. As a result, females may stop feeding themselves in advanced stages of gravidity (Appel et al. 2011). Although the marsupium of conglobating species does not protrude as in non-conglobating species, in the last days of the incubation of manca it can prevent perfect conglobation. The length of the breeding period can be shortened due to the presence of predators (Castillo and Kight 2005), which is indirect support that conglobation is an antipredatory strategy. Additionally, due to the smooth surface of the conglobated isopod, it is more difficult for predators to find a suitable place for attack (Řezáč et al. 2008).

Conglobation is usually triggered by external stimuli, such as strong vibrations or pressure (Horváth et al. 2019). Cividini and Montesanto (2018a) documented that *Armadillo officinalis* Duméril, 1816 responded to substrate vibrations by conglobation. Even against larger, visually oriented predators, conglobation can be a useful adaptation since a ball-like body can roll away and disappear into leaves or debris. This is more important for non-perfect conglobation (typical of the genus *Cylisticus*), because the uropods and antennae are not well protected. Anecdotally, it is cruelly ironic that the typical ball-like shape of a defending *A. vulgare* was the only reason that humans ate them (giving the name of “pill-bug” to all conglobating terrestrial isopods) – its antipredatory strategy was, in this case, a reason for higher predatory pressure (Duméril 1816).

Although intraspecific variability in the use of tonic immobility in the “clinger” species *P. scaber* is high (Tuf et al. 2015), conglobation used by rollers is more constant

(Matsuno and Moriyama 2012; Cazzolla Gatti et al. 2019). In addition to its function for protection against predators, terrestrial isopods can use conglobation to limit water loss (Smigel and Gibbs 2008).

Not to be alone

If a prey species is distributed homogenously, it is easier for a predator to encounter prey frequently and eat ad libitum; therefore, a very simple antipredatory strategy is for prey to be grouped together. Aggregation into groups is considered an evolutionarily successful response to predator pressure, ambient temperature, and water deficits (Broly et al. 2013). The advantages of living in aggregations were described by Allee (1926), and positive density dependency, or the positive correlation between population density and individual fitness, is called the “Allee effect” (Krause and Ruxton 2002). As Krause and Ruxton (2002) pointed out, aggregative defensive behaviour is a common response to the risk of predation, and is widespread among a diverse range of animal groups.

One of the basic characteristics of this type of defensive behaviour is the predator confusion. People who have tried to collect aggregated isopods can confirm that individually handling them can give the majority of isopods enough time to disappear. Even a skilled predator is not capable of eating all individuals in a group. The size of the group has an inverse correlation with the probability that a particular individual in the group will be attacked; i.e., the larger the group, the less likely it is for an individual to be attacked. Actually, a higher visibility of large groups of prey, i.e., a higher attack rate per group, is less important for each individual than is the much lower probability of being eaten while “hiding” in large groups (Krause and Ruxton 2002).

In aggregations, information about an approaching attack can be transmitted from individuals who did observe the danger to those who have not yet noticed it. Such behaviour was documented in *A. officinalis*, which can produce substrate vibrations to warn neighbouring individuals (Cividini et al. 2020). In addition, aggregation can intensify the effect of individual defence mechanisms, such as repulsive secretions or necromones (chemical compounds released by dead terrestrial isopods, cf. Yao et al. 2009), and thus functions as a shared defence behaviour (Broly et al. 2013). The study of Cividini and Montesanto (2018a) proved that the isopod's response to micro-vibrations leads to a greater number of aggregates, considering that micro-vibrations can warn for an impending danger. Aggregation behaviour in terrestrial isopods is thoroughly studied (Broly et al. 2012, 2013, 2014, 2016; Broly and Deneubourg 2015; Pogson 2016); however, a comprehensive study of the impact of aggregation on predation risk in terrestrial isopods is yet to be done.

Only one terrestrial isopod, *A. officinalis*, is known to produce sounds that are audible to humans. This sound is produced by stridulation through a ledge of scales situated on the propodus of the fourth and fifth pereopod (Caruso and Costa 1976). This feature is present in both sexes (Taiti et al. 1998) from the early stages of development onwards (Montesanto 2018) and occurs in all species of *Armadillo* (Schmalfuss 1996).

Although terrestrial isopods do not have a sense of hearing, they can register substrate-borne vibrations caused by their stridulation (Cividini and Montesanto 2018a, b). *Armadillo officinalis* responds to substrate vibrations by conglobation or by deviating from the source of vibration, although juveniles usually conglobate (Cividini and Montesanto 2018c). This response may be caused by the perception that these vibrations are a sign of danger (Zimmerman and Kight 2016). Escape behaviour in response to vibrations was also noted in *A. vulgare* by Moriyama (2004), and *P. laevis* systematically alternates its turns in a T-maze in response to vibrations when not habituated to the vibrations beforehand (Houghtaling and Kight 2006). Although no sensory receptor in terrestrial isopods has been reported yet, the high sensitivity of *A. vulgare*, *P. laevis* as well as *A. officinalis* to vibrations suggests its presence.

Additionally, substrate-borne vibrations induced by stridulations can be a strategy of intraspecific communication. The pill bug *A. officinalis* can probably warn other individuals of imminent danger and adverse conditions, and thus ensures a higher survival rate of neighbouring individuals (Cividini et al. 2020). Indeed, its response to micro-vibrations is an intensification of aggregation behaviour (Cividini and Montesanto 2018a). Perhaps *A. officinalis* can also use stridulation during mating to convince females to uncoil and mate, as the morphologically similar deaf giant pill millipedes (Sphaerotheriida) do (Wesener et al. 2011); however, this topic has not been studied yet.

Stridulation could also work as an antipredatory strategy. In giant pill millipedes (Sphaerotheriida) this function of stridulation was reported more than a hundred years ago (Gravely 1915) as protecting *Arthrosphaera aurocincta* Pocock, 1899 against reduviid bugs of the genus *Physorhynchus*, but without a description of the mechanism. As in the case of *A. officinalis* (Cividini et al. 2020), these millipedes are only able to stridulate during the conglobation (the antipredator behaviour mentioned above). Thus, stridulation, as a secondary form of defence, can be used to discourage predators more effectively. These millipedes stridulate in response to handling (Gravely 1915) in a similar way to *A. officinalis*.

Defensive sounds could similarly be aposematic; that is, they could be the acoustic counterparts of visual aposematic signals, differing only in the way that they can fulfil their function both in daylight and in darkness. Defensive stridulation is known from many species of arachnids, myriapods, insects, as well as crustaceans. Usually, these species use stridulation to warn predators against inflicting an attack on poisonous scorpions, spiders, harvestmen, centipedes, or mutilid wasps (Iorio 2003; Pomini et al. 2010; Esposito et al. 2018; Gall et al. 2018; Stidham 2019). The sounds could therefore fulfil the same role as gaudy colouration (Rowe 2002). Stridulation also can be mimicked e.g., harmless spiders which mimic mutilid wasps in size, colour, and stridulation (Pekár et al. 2020), though this is rarely used as part of the behaviour of a mimicry model. Although we do not have any evidence about a possible mimicry model for *A. officinalis*, which can teach predators to avoid dangerous vibrating prey, stridulation can be effective as a defensive behaviour without a painful experience for the predator. Vibrating can cause prey to be dropped, resulting in its loss on the soil surface. The ball-like shape of conglobated stridulating *Armadillo* makes it predestined to roll away. However, experiments with experienced and naïve predators of *A. officinalis* should be done to confirm this theory.

Not to be edible

When a terrestrial isopod is found and recognised by a predator, there are some other possible strategies it can use to avoid being consumed. A widespread strategy to repulse predators is the use of excretions from the defensive glands. A detailed description of the glands of terrestrial isopods, which are diverse and numerous, was done by Gorvett (1946, 1951, 1952, 1956). There are several different kinds of glands, occurring in almost every part of the body. As pointed out by Gorvett (1951), lobed glands are the largest, as well as the most interesting, of the tegumental glands. They have numerous long ducts that end in separate external openings along the lateral plates and uropods. After strong stimulation, visible droplets of a viscous, smelly secretions appear from these openings. The distribution and size of these glands were studied by Herold (1913), who explained the reduction of their function in some myrmecophilous species, that are defended by tenant ants. On the other hand, the reduction of the function of these glands can be a reason for ant adoption of some myrmecophilous species, as they are chemically insignificant for them (Parmentier 2016), and difficult to recognise in an ant nest.

The substance of lobed glands has a proteinaceous composition with a secretion that is not associated with the hormonal or nervous system; it is instead probably caused by the contraction of adjacent muscles. The stimulation must be very violent: simple shaking or squeezing of the animal does not affect gland secretion, in general (Fig. 1). Gorvett (1956) experimentally confirmed that droplets of secretion appeared



Figure 1. A male of *Porcellio bolivari* Dollfus, 1892 releasing proteinaceous secretion from the uropods (string with droplets) (photograph Adrián Purkart).

after a spider bit a woodlouse on the leg, or after an experimental pinprick. It is apparent, then, that the function of these glands is to produce defensive secretions against spiders that belong to the most significant predators of terrestrial isopods. The pores of the lobed glands are in an optimal position for maximal effect against attacking spiders (Gorvett 1956), centipedes (Paris 1963), or ants (Deslippe et al. 1996). After a spider bites, using its chelicerae, lobed glands begin to secrete defensive secretions, causing the predator to retreat in order to clean its mouth parts. This observation is supported by the fact that terrestrial isopods with missing parts of the uropods or lateral parts are frequently found in nature. This may be caused by shrews' (Brereton 1957) or scorpions' (Herold 1913) incomplete attacks (Gorvett 1956).

Instead of discouraging a predator from an attack using repellent glands, terrestrial isopods may display unpalatability from a distance in order to avoid risking damage. One way to do this is by using a warning aposematic colouration. This strategy is widely adopted by different insects, millipedes (Marek and Bond 2009), and arachnids. Warning colours (usually black combined with red, orange, or yellow) can warn predators of their unpalatability through inherited neophobia or learned avoidance for these colours (Vickers et al. 2021). So far, we have only anecdotal evidence for the aposematic function of colouration in terrestrial isopods. Levi (1965) compared bright red spots on *Armadillidium klugii* Brandt, 1833, the millipede *Glomeris pulchra* CL Koch, 1847, and the widow spider *Latrodectus tredecimguttatus* (Rossi, 1790), all living syntopically near Dubrovnik. All of these species defend themselves chemically, that support their Mullerian mimicry system against attacks from thrushes, gallinaceous birds, nocturnal mammals, and geckos (Schmalfuss 2013).

There are dozens of species with an ostentatious colouration (Fig. 2). Yellow and white spots are quite common in the genus *Porcellio*, but “black” and white patterns (more precisely dark violet-brown and white patterns) were reported for several non-related litter-dwelling species in western Africa (Schmalfuss and Ferrara 1982). Its antipredation function was suggested to not only be a warning colouration, but also a cryptic colouration that dissolves the body outline (Schmalfuss and Ferrara 1982). Some species are capable of polychromatism (Achouri and Charfi-Cheikhrouha 2009), in which individuals with different colour patterns coexist in the same population. Such variability in colour pattern can be useful for individuals having less frequent variations since predators are generally attracted to the most frequent prey type due to perceptual learning (“search image”, see Punzalan et al. 2005). Thus, polychromatism is a mechanism of negative frequency-dependent selection, where a rare morph prey experiences a higher survival rate than those of more common types (Hughes and Mather 1986).

Warning colouration works only against visual predators, but there could be another possible method to warn other predators: spines on the dorsal surface of terrestrial isopods. Extravagant pin-like or blade-like spinulation is typical for several species of the families Armadillidae (species of the genera *Pseudolaureola*, *Calmanesia*, *Echinodillo*, *Tridentodillo*, *Globarmadillo*, *Polyacanthus*), Eubelidae (*Panningillo*), and Delatorreidae (*Pseudarmadillo*, *Acanthoniscus*). All of these species are able to conglobate, and are of small size (~ 1 cm at maximum).



Figure 2. Colourful species of terrestrial isopods: **A** *Armadillidium wernerii* Strouhal, 1927 **B** *Porcellio dubosqui* Paulian de Félice, 1941 **C** *Armadillidium gestroi* Tua, 1900 **D** *Porcellio haasi* Arcangeli, 1925 **E** “*Merulanella*” sp. 1 from Thailand **F** “*Merulanella*” sp. 2 from Thailand (photographs Adrián Purkart).

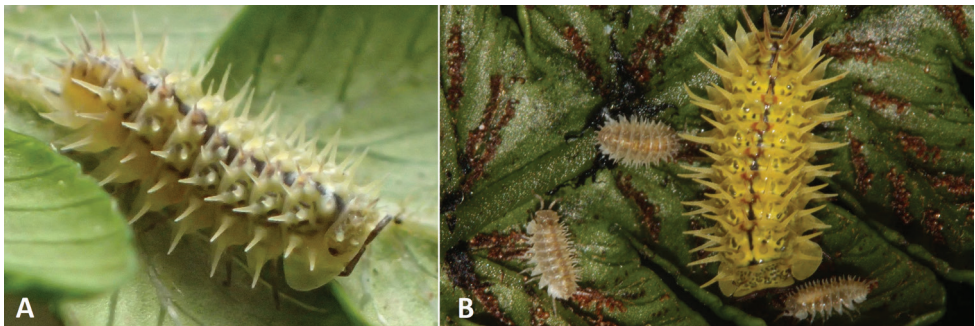


Figure 3. The spiky yellow woodlouse *Pseudolaureola atlantica* (Vandel, 1977), endemic to St. Helena Island **A** its bright coloration and spines can serve as protection from potential predators (photograph Amy-Jayne Dutton, St Helena National Trust) **B** juveniles of *P. atlantica* are well protected as soon as they leave the marsupium, and remain close to their mother (photograph Phil Lambdon).



Figure 4. Threatening posture of a male of *Porcellio magnificus* Dollfus, 1892 resembling the threatening posture of a syntopic *Buthus* scorpion (photographs Adrián Purkart).

Long spines on terrestrial isopods can also, theoretically, be useful against predators (Fig. 3). The spiky yellow isopod, *Pseudolaureola atlantica* (Vandel, 1977), lives on tree fern leaves, and, despite its vivid colour, softens its body outline with its long spines (Dutton and Pryce 2018). The function of long spines has not been studied to date, but it is assumed that spines protect terrestrial isopods against swallowing by geckos and other lizards, frogs, and birds, as well as from ants and other smaller predators (Schmalfuss 1975). Among millipedes, soft-bodied bristly millipedes (Polyxenida) without defence glands wear lateral tufts of setae and use them against ants (Eisner et al. 1996). Perhaps long spines on some small conglobating terrestrial isopods can prevent them against grasping by ant mandibles. A similar function can be ascribed to shorter and stronger thorns, which are more frequent among terrestrial isopods. Strong thorns can also protect larger soil-dwelling terrestrial isopods. The genus *Hemilepistus* (e.g., *Hemilepistus aphganicus* Borutzky, 1958) has strong thorns on the anterior part of the head and posterior margin of the first four pereonites. The function of this armature is to plug the entrance of its burrow, and to protect the individuals inside against small predators and other intruders (Schmalfuss 1975).

A threatening posture, as seen in scorpions, bird-spiders, or centipedes, is another warning signal that protects prey from predators (Kronmüller and Lewis 2015). Several large species of *Porcellio* from the Iberian peninsula are capable of bending the posterior part of their body upward, with their long uropods targeted forward, and with widely outspread antennae (Fig. 4) when they are disturbed. This posture resem-

bles the posture of a scorpion. The Iberian peninsula is inhabited by at least a dozen species of the scorpion genus *Buthus* (Teruel and Turiel 2020), all of which have pale orange-brown colour and thin chelae. *Porcellio magnificus* Dollfus, 1892 is of orange colour and readily takes this posture. Although we lack supporting experimental evidence about whether this behaviour can avert predator attacks, it is known that even some lizards from Southern America (Brandão and Motta 2005) and Asia (Autumn and Han 1989) are known to be scorpion mimics. A scorpion-like threatening posture is not possible to use in a tight shelter or a burrow but only at the surface. Due to probable nocturnal activity, as well as its only superficial resemblance, it is plausible to suppose that *Porcellio* can use this posture during moonlit nights against their predators. However, this topic has not been studied yet.

Conclusions

We have summarised what is known about the antipredatory strategies of terrestrial isopods. Some anatomical and behavioural traits should be classified as pre-adaptations because they help to solve other challenges of woodlouse life, such as the reduction of water loss. Examples include conglobation and aggregation, as well as clinging (Warburg 1993). Additionally, some proposed strategies can execute different functions, e.g., long spines can be used as tactile sensors or for collecting water from fog as well as defence mechanism. On the other hand, the significance of the jumping or scorpion-like threatening posture is difficult to understand without considering the behaviour of its predators. Some of the strategies mentioned above, such as escape by turn alternation, tonic immobility, production of repelling secretions, or conglobation were studied with their respect to predation pressure, but most of them were only suggested to have this protective function against predators. These knowledge gaps deserve attention in future research.

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Immune priming in *Armadillidium vulgare* against *Salmonella enterica*: direct or indirect costs on life history traits?

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Abstract

Invertebrate immune priming is defined as an enhanced protection against secondary pathogenic infections when individuals have been previously exposed to the same or a different pathogen. Immune priming can be energetically costly for individuals, thus impacting trade-offs between life-history traits, like reproduction, growth, and lifetime. Here, the reproductive cost(s) and senescence patterns of immune priming against *S. enterica* in the common woodlouse *A. vulgare* (Crustacea, Isopoda) were investigated. Four different groups of females were used that either (1) have never been injected (control), (2) were injected twice with *S. enterica* (7 days between infections), (3) were firstly injected with LB-broth, then with *S. enterica*, and (4) females injected only once with *S. enterica*. All females were allowed to breed with one non-infected male and were observed for eight months. Then, the number of clutches produced, the time taken to produce the clutch(es), the number of offspring in each clutch, the senescence biomarkers of females, and parameters of their haemocytes were compared. The result was that immune priming did not significantly impact reproductive abilities, senescence patterns, and haemocyte parameters of female *A. vulgare*, but had an indirect effect through body weight. The lighter immune primed females took less time to produce the first clutch, which contained less offspring, but they were more likely to produce a second clutch. The opposite effects were observed in the heavier immune primed females. By highlighting that immune priming was not as costly as expected in *A. vulgare*, these results provide new insights into the adaptive nature of this immune process.

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Keywords

Crustacea, haemocytes, immune priming, isopod, reproduction, senescence, survival, trade-off

Introduction

Because fighting pathogens is a real challenge for all living organisms, they have developed an important and complex biological process, the immune system (Siva-Jothy et al. 2005; Danilova 2006), which is commonly divided into the innate immunity, which is found in all organisms and is based on non-specific recognition of intruders (Medzhitov and Janeway 2000), and the adaptive immunity, which is observed only in jawed vertebrates and allows the remembering of previous encounters with pathogens to prepare the immune response for a second exposure. Adaptive immunity mainly rely on specialised cells, the lymphocytes (Cooper and Alder 2006). Because invertebrates only have the innate immunity, it has long been thought that they were not able to express memory-like features following subsequent infections with pathogens (Janeway and Medzhitov 2002; Cooper and Eleftherianos 2017). Recent findings reported the other way around: in more than 40 invertebrate species, individuals improve their survival abilities upon a second pathogenic infection when they have been previously exposed to the same, or another, pathogen (Little and Kraaijeveld 2004; Kurtz 2005; Milutinović and Kurtz 2016; Netea et al. 2019). In invertebrates, this immune process is called “immune priming”. Immune priming can last for a few days to several months depending on the species (Milutinović and Kurtz 2016) and is mediated by three, non-exclusive, major mechanisms: (1) the recalled immune response, involving a first immune response that returns to basal levels before the second exposure to pathogen, then a second enhanced immune response with the same immune activity (Contreras-Garduño et al. 2016); (2) the immune shift, that firstly involves one type of immune activity (e.g., cellular encapsulation), then a different kind of immune activity during the second infection (e.g., humoral expression of antimicrobial-related genes; Pinaud et al. 2016); and (3) the sustained immune response, which lasts from the first to the second infection event (reviewed in Coustau et al. 2016; Melillo et al. 2018).

Although immune priming is advantageous in terms of survival ability, its expression could be costly for individuals, particularly when it relies on the long-lasting sustained immune response (Moret 2003; Contreras-Garduño et al. 2014, 2019; Coustau et al. 2016; Shikano et al. 2016; Khan et al. 2019). According to the theory of life-history traits, organisms must allocate their limited energy to different biological functions by making trade-offs. Individuals which invest a lot of energy in reproduction during their early life may have lower survival abilities (Descamps et al. 2006; Lemaître et al. 2015). On the opposite side, individuals investing in their somatic maintenance (including pathogen clearance) may have less energy for other biological functions, decreasing thus their reproductive ability (Rolff and Siva-Jothy 2003; Schmid-Hempel 2005; Luu and Tate 2017). In *Anopheles gambiae* (Giles 1902), immune primed fe-

males which clear the second infection of *Plasmodium berghei* (Vincke & Lips, 1948) do not produce eggs, and those which do not successfully eliminate *P. berghei* exhibit a lower hatching rate compared to non-primed females (Contreras-Garduño et al. 2014). In addition, the immune responses of invertebrate individuals sometimes involve cytotoxic compounds (e.g., phenoloxidase products, reactive oxygens species), which can damage the cells and tissues of the host, especially if the immune response is systemic (Moret 2003; Sadd and Siva-Jothy 2006). However, the investment in immune functions can sometimes enhance the reproductive abilities of individuals, by the terminal investment strategy (Adamo 1999; Duffield et al. 2017; Luu and Tate 2017). The terminal investment strategy refers to the direct allocation of the remaining energy in reproduction when the individual's probability of dying is expected to increase (Creighton et al. 2009; Duffield et al. 2017). This strategy can also be triggered by the senescence patterns of individuals because natural ageing or advanced senescence caused by environmental/physiological stress results in somatic deterioration and reduced lifetime, like immune response can do (Comfort 1964).

Nevertheless, several studies did not observe the impact of repeated infections with pathogens on the reproductive abilities of individuals, within the same or the subsequent generations (Faria et al. 2015; Gupta et al. 2016; Prakash et al. 2022). In a recent experimental evolution study, *Tribolium castaneum* (Herbst, 1797) lineages that evolved with two consecutive infections with inactivated and living *Bacillus thuringiensis* (Berliner, 1915) for 14 generations displayed lower reproductive costs compared to lineages that evolved only with inactivated *B. thuringiensis* (Prakash et al. 2022). Females of *Tenebrio molitor* (Linnaeus, 1758) previously challenged with *Arthrobacter globiformis* (Conn & Dimmick, 1947) or *B. thuringiensis* produce as many eggs as the non-primed females, and these eggs have higher hatching rates (Dhinaut et al. 2018). These experiments highlight that immediate reproduction is not always influenced by the increased immune protection across generations. Most empirical studies that investigated the impact of immune priming on the reproduction of females analysed their reproductive abilities after the first infection and on the next generation. Although these results provide important knowledge on the evolutionary trade-offs, with reproduction resulting from the first immune stimulation, we are lacking knowledge about the reproductive cost(s) of females that survived the first immune stimulation and the second, lethal pathogenic infection with living pathogens. We also lack information on whether immune priming and the following reproductive event(s) affect the senescence pattern of individuals, although they should indicate the adaptive features of such immune process.

Among the numerous species in which immune priming has been observed, the common woodlouse *Armadillidium vulgare* (Oniscidea, Isopoda, Crustacea) is an appropriate model to investigate this issue. *Armadillidium vulgare* (Latreille, 1804) mount an immune priming response with two subsequent infections of living *Salmonella enterica* (Theobald Smith, 1855) injected seven days apart (Prigot-Maurice et al. 2019, 2021). The underlying mechanism is expected to be a sustained immune response of primed individuals, which display long-lasting, higher viability of haemocytes com-

pared to non-primed individuals (Prigot-Maurice et al. 2019). This assumption is supported by the persistence of *S. enterica* in the haemolymph of the primed individuals between the infections. Although primed individuals would die faster than those receiving only one injection, this study shows that they are even more able to survive and deal with very high titres of pathogenic bacteria (Prigot-Maurice et al. 2019). Hence, the energetic costs of mounting immune priming and simultaneously repairing the tissues damaged by the persistent infection of *S. enterica* would be very high in this host-pathogen system. The reproductive events in female of *A. vulgare* also require important energetic resources (Warburg et al. 2001). To reproduce, females develop exoskeletal extrusions which form a ventral brood pouch, named marsupium. Females oviposit and incubate their eggs in the marsupium throughout embryogenesis for ca. a month (Surbida and Wright 2001). On average, females produce one or two marsupia (i.e., one or two clutches) per year, during the reproductive season (Lawlor 1976; Dangerfield and Telford 1995). This extensive maternal care partly determines the fecundity of females, because larger females produce larger marsupium in which they can incubate more eggs than smaller females (Sutton et al. 1983; Antoł and Czarnoleski 2018; Durand et al. 2018). *Armadillidium vulgare* exhibits indeterminate growth, allocating energy in growth throughout its lifetime. In this species, the size of individuals is closely correlated to their weight, and the largest and heaviest individuals are usually the oldest (Depeux et al. 2020b). In natural populations, the average lifespan of *A. vulgare* is two years (Paris and Pitelka 1962). Females in the field reproduce until three years old (Dangerfield and Hassall 1992), although most individuals die at approximately the age of one year old, following the first reproductive season (Paris and Pitelka 1962). In addition to these biological features, *A. vulgare* live in an environment rich in microbial density and diversity (Warburg et al. 1984; Broly et al. 2013; Ranjard and Richaume 2001; Zimmer 2002), where the risk of exposure to pathogens related to its lifespan is expected to be high (Little and Kraaijeveld 2004). Hence, the use of *A. vulgare* opens up the possibility to easily observe evolutionary trade-offs between somatic maintenance and reproductive investment, with the main objective being the exploration of adaptive features of immune priming. Recently, several biomarkers have been identified to estimate the cellular senescence patterns in *A. vulgare* (Depeux et al. 2020b). These biomarkers allow us to determine the senescence patterns of individuals of the same age. They may thus provide important clues about the cost(s) of maintaining survival and/or reproductive ability under stressful conditions, like infections with pathogens.

In this study, we explored the impact of immune priming with *S. enterica* on the reproductive ability and the resulting senescence patterns of *A. vulgare*. Our objectives were: (1) to test whether mounting an immune priming response affects the reproduction of females that successfully survived two consecutive infections with living *S. enterica*, and (2) to explore to what extent immune priming and reproduction change the senescence patterns of individuals, by using two senescence biomarkers: the β -galactosidase activity and the size of the viable haemocytes (described in Depeux et al. 2020b). Because reproduction is known to negatively affect the immune system (Lawniczak et al. 2007), we also (3) compare the total concentration and viability of haemocytes after the last reproductive event of females. To

do so, we used females firstly injected (i.e., primed) either with a low living dose of *S. enterica*, sterile LB-broth, or non-primed females. Seven days later, we injected all these females with LD₅₀ of *S. enterica*. Since the fecundity of terrestrial isopod females is correlated with their size (Sutton et al. 1983; Antoł and Czarnoleski 2018; Durand et al. 2018) and immune responses may decrease the growth and/or body weight of the females (Moret 2006; Bascuñán-García et al. 2010; Kelly 2011), we weighed the females that survived the sublethal infection of *S. enterica* and allowed them to mate with one virgin, non-injected male. We also added females that have never been injected with *S. enterica* (control group) but maintained under the same experimental conditions as the females in the other treatments. To compare the reproductive cost of surviving females that mounted immune priming or not, we measured the probability of producing one or two clutches, the time taken to produce these clutches, and the number of viable offspring in each clutch. After the reproductive event(s), we analysed the β -galactosidase activity and the haemocyte parameters (size of viable haemocytes, concentration, and viability of haemocytes) of all females.

Materials and methods

Biological model and bacterial cultures

In this experiment, we used the same *Armadillidium vulgare* line used in the study of Prigot-Maurice et al. (2019). The virgin females with an age of one year (± 2 months) came from laboratory cross-breeding of individuals initially sampled at Helsingør, Denmark (1982). During the breeding period, females were kept in moistened potting mix supplied with linden leaves and carrot slices ad libitum in 10 \times 30 cm boxes, under natural photoperiod and room temperature.

To perform the infections, we used the *Salmonella enterica* serovar *typhimurium* J18 strain (Verdon et al. 2016). The cultures of *S. enterica* were performed as described in Braquart-Varnier et al. (2015). Briefly, the *S. enterica* strain came from one frozen glycerol stock, streaked on Luria-Bertani Broth plates (25 g.L⁻¹ of LB base, Invitrogen 12795-027 supplemented with 15 g.L⁻¹ of agar-agar, Fisher BioReagents, BP1423-2) at 37 °C overnight. One Colony Forming Unit (CFU) was then added to 5 mL of liquid LB broth at 37 °C, 180 rpm overnight. 100 μ L of this *S. enterica* culture were grown in 3 mL of fresh LB broth under the same conditions to reach an optical density of 0.8 (600 nm). 1 mL was centrifugated (2 min, 4 °C, 13,000 g) and the bacterial pellet resuspended in 100 μ L of LB broth. This first tube contained 10⁶ *S. enterica* for 100 nL of injection (LD₅₀ dosage). Serial dilutions were then achieved to obtain the dosage for the first injection (10³ *S. enterica* for 100 nL). To control the quantity of injected *S. enterica*, we diluted four times more the first tube, reaching the concentration of 1 bacterium per μ L. We plated 100 μ L of this solution onto LB agar plate and we counted the number of CFU after an overnight culture at 37 °C.

Experimental design

Firstly, we performed the priming procedure on three females' treatments: either primed (i.e., primo-injected) with the low dose of living *S. enterica* (SAP, for *S. enterica*-primed), with sterile LB broth (LBP, for LB-primed) or without priming injection (NP, for non-primed; Fig. 1). We added a fourth treatment in which the females have never been injected (control females). We used a total of 123 females, including 32 SAP, 33 LBP, 33 NP, and 25 control. Seven days after the priming procedure, SAP, LBP and NP females were all injected with a LD₅₀ of *S. enterica*, and their survival rates were monitored for 22 days. Surviving females (SAP = 26, LBP = 27, NP = 20, control = 25) were weighted and placed onto a box (5×8cm) with one virgin non-injected male (Fig. 1). Each pair of individuals were kept on moistened potting mix with linden leaves and carrot slices ad libitum under a stimulating photoperiod (18:6 D/N) at 21 °C.

Every three days for ca. eight months, we measured the survival rate and the physiological states of all females by observing their ventral faces. The females that were about to lay eggs developed a marsupium following a parturial moult, which is observable under a binocular loupe (Moreau and Rigaud 2002). Once females were ready to deliver their offspring, they were placed alone in a box on moist paper. For each female, we counted the number of clutches (one or two), the number of days that they took to produce each clutch (i.e., from the contact with the male to the delivery of offspring; or the time between the first and the second clutch), the number of offspring produced in each clutch and the total number of produced offspring (Fig. 1). After the second

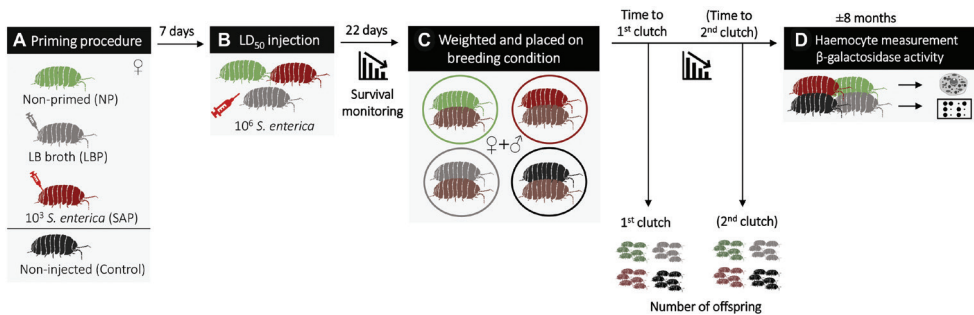


Figure 1. The experimental procedure **A** the priming procedure was to inject females either with a low dose of living *S. enterica* (SAP, in red) or sterile LB Broth (LBP, in grey). The non-primed (NP, in green) females did not receive the first injection **B** SAP, LBP, and NP females received the second, LD50 injection of living *S. enterica*. Control females (in black) were never injected **C** all females (SAP, LBP, NP, control) were allowed to reproduce individually in a box with one virgin, non-injected male (brown woodlouse). We checked the survival rates of females, the number of clutches (1 or 2), the time to produce each clutch (number of days), and the number of offspring in each clutch **D** regularly, we sampled and dissected females that produced the second clutch to analyse haemocytes and β -galactosidase activity. Brackets indicate that not all females produced a second clutch. Approximately eight months later, we waited for the last females to produce their second clutch, and then sampled and dissected the remaining females that produced only one clutch.

clutch (when it occurred), we washed the females (0.28% NaClO then water) and measured the viable haemocyte size, viability (% of living cells), and concentration. We dissected the nerve cord to quantify the β -galactosidase activity. Because not all females produced a second clutch, we decided to sample and dissect the females that had only one clutch at the same time we did this for the last females which produced the second clutch (ca. eight months after the beginning of the experiment; Fig. 1).

Priming procedure and LD₅₀ injection

The priming procedure with *S. enterica* was performed as described in Prigot-Maurice et al. (2019). Briefly, females were washed (0.28% NaClO then water) and injected dorsally between the 6th and 7th pereon segment under sterile conditions, using a Drummond TM Nanoject (3–000–205A). Injections were performed by two successive injections of 50 nL, either with $10^3 \pm 1.10^2$ living *S. enterica*, or sterile LB Broth. All females (SAP, LBP, NP, and control) were individually isolated in a plastic box, on moist paper without food. We controlled the injected dosage as described above. Seven days later, the SAP, LBP, and NP females were injected with $10^6 \pm 1.10^5$ living *S. enterica* in 100 nL of LB broth (i.e., LD₅₀, dosage to kill 50% of individual in seven days) following the same method as for the priming procedure. Females were replaced on their box, and we monitored their survival rates (i.e., immune priming protection) every eight hours for seven days. We adjusted the humidity of each box daily. Then, females were placed alone on moistened potting mix with linden leaves and carrot slices ad libitum for fifteen days and we added the virgin, non-injected male.

Haemocyte analysis

After their second clutch, females were washed (0.28% NaClO then water). The total haemocyte concentration (number of haemocytes per μ L of haemolymph, regardless of the haemocytes' type), the viability of haemocytes (% of living haemocytes), and the size of viable haemocytes (μ M) were measured as described in Sicard et al. (2010). Three μ L of haemolymph were sampled by piercing the females in the 6th tergite with a sterile needle and diluted in 15 μ L of MAS solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7; Herbinière 2005). We added 6 μ L of Trypan blue (0.4%) and 10 μ L of the resulting sample were deposited in the counting chamber of the automated cell counter Countess™ Version B (Invitrogen). We waited for the last females to produce their second clutch (ca. eight months) to sample and dissect females which produced only one clutch.

β -galactosidase activity

After collecting their haemolymph, all females used for haemocyte analysis were dissected in Ringer solution (135 mM sodium chloride, 2 mM potassium chloride, 2 mM calcium chloride, 2 mM sodium bicarbonate) to collect their nerve cords. The

β -galactosidase activity was measured as described in Depeux et al. (2020b). Briefly, each nerve cord was deposited in 300 μ L of Lyse buffer 1X (5 mM Chaps detergent, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine, and 0.25 mM PMSF, pH 6), ground manually, and centrifugated for 30 min, 15 000 g, 4 °C. The supernatant was collected and proteins were assayed using the Bicinchoninic Acid Assay (Thermo Fisher Scientific) and standardised at 0.04 mg.mL⁻¹ to perform the β -galactosidase activity assay on the same titres of proteins. Subsequently, 100 μ L of each protein sample was added to the MUG reagent solution (4-methylumbelliferyl-D-galactopyranoside) in a 96-well microplate. The fluorescence produced by the synthesis of 4-methylumbelliferone (4-MU) was measured by the multimode Mithrax microplate reader (LB940 HTS III, excitation filter: 120 nm, emission filter: 460 nm; Berthold Technologies) for 120 minutes. Two technical replicates were set up for each sample to obtain the result by averaging the replicates' values.

Statistical analysis

All statistical analysis were performed with RSTUDIO (v.1.4; R Core Team 2017). We compared the survival rates of females after the LD₅₀ and during the reproductive period by using two global mixed effects Cox proportional hazard regression models, built with coxme package (Therneau et al. 2003). We entered the females' treatment (SAP, LBP, NP, control) as fixed effect, and Hazard Ratios (HR) were estimated thanks to the instantaneous risks of death between NP or control and other females' treatments (SAP, LBP, and NP).

Body weight differences of females before reproduction were tested with a linear mixed effects model built with lme4 and car package (Fox and Weisberg 2011; Bates et al. 2014), including the treatment as fixed effect.

Concerning the first reproductive event, we tested the probability of producing the first clutch with one generalised linear mixed effects model with binary logistic regression (i.e., 1-0; Harrell 2015). As the body weight of females is known to influence their fecundity (Sutton et al. 1983; Antol and Czarnoleski 2018; Durand et al. 2018), we included the weight, the treatment, and their interaction as fixed effects. The time to produce the first clutch (i.e., number of days from the first contact with male to the delivery of offspring) and the number of offspring of the first clutch were modelled in two linear mixed effects models including the weight, the treatment, and their interaction as fixed effects. For the second reproductive event, we used three models like those concerning the first reproductive event. Since the first reproduction can influence the second reproduction by energy investment, we added the number of produced offspring in the first clutch as fixed effect, with the treatment, the weight and the interaction between the treatment and the weight.

The total number of offspring (first and second clutches included) was analysed with one linear mixed effects model including the treatment and the weight as fixed effects.

The haemocyte concentrations (number of cells per μ L of haemolymph), the size of viable haemocytes and β -galactosidase activity were analysed using linear models

with Gaussian distribution, and viability (proportion of viable haemocytes) using one generalised model with Binomial distribution (Harrison et al. 2018). Since the haemocyte parameters and senescence biomarkers were analysed both in females having one or two clutch(es), we used four mixed effects models, including the treatment, the number of clutches and the total number of offspring (first and second clutches included) as fixed effects. To deepen the interdependence of treatments and reproduction, we only allowed interactions of the treatment with the total number of offspring, and the treatments with the number of clutches.

For all models (i.e., survival, weight, probability of producing the first and second clutches, time to produce these clutches, the number of offspring in each clutch, haemocyte parameters and β -galactosidase activity), we entered the experimental replicates as random factor. This factor allows to correct the non-independence of samples within the same replicate of treatment (Harrison et al. 2018). Whether the treatment influenced the considered variable, we compared the pairs of means between each treatment by using Tukey adjustment (lsmeans packages; Lenth 2016). When interactions with treatment and weight were significant in our models, we performed the Pearson's correlation test for each treatment, to obtain the effect of weight in each treatment.

The R script and the datasets used to perform our analysis are available on the open access repository Mendeley Data <https://data.mendeley.com/datasets/gd24nvnvcvf/2>

Results

Survival abilities

The treatment had a slight effect on the survival abilities of females after the LD₅₀ injection and before reproduction ($X^2 = 5.17$, $df = 2$, $p = 0.07$; Fig. 2A). SAP and LBP females tended to better survive than NP females ($p = 0.06$ for each, Suppl. material 1: Table S1), with significant 59% lower risk of death for each (LBP: HR = 0.41, 95% CI = [0.15-1.1]; SAP: HR = 0.41, 95% CI = [0.16-1.1]). No difference in survival rates was observed between SAP and LBP females (Fig. 2A, Suppl. material 1: Table S1). During the reproductive period, the survival rates of females were not influenced by their treatment ($X^2 = 0.53$, $df = 3$, $p = 0.91$; Fig. 3). Whatever the number of infections with *S. enterica*, injected females had same survival abilities with control females that have never been injected (Fig. 2B, Suppl. material 1: Table S1).

Body weight of females

The weight of females after the second infection with *S. enterica* (before reproduction) was influenced by their treatment ($X^2 = 8.05$, $df = 3$, $p = 0.04$; Suppl. material 1: Fig. S1, Table S2). Even though the pairs of comparisons between females of the different treatments didn't show significant effects, control females had an average

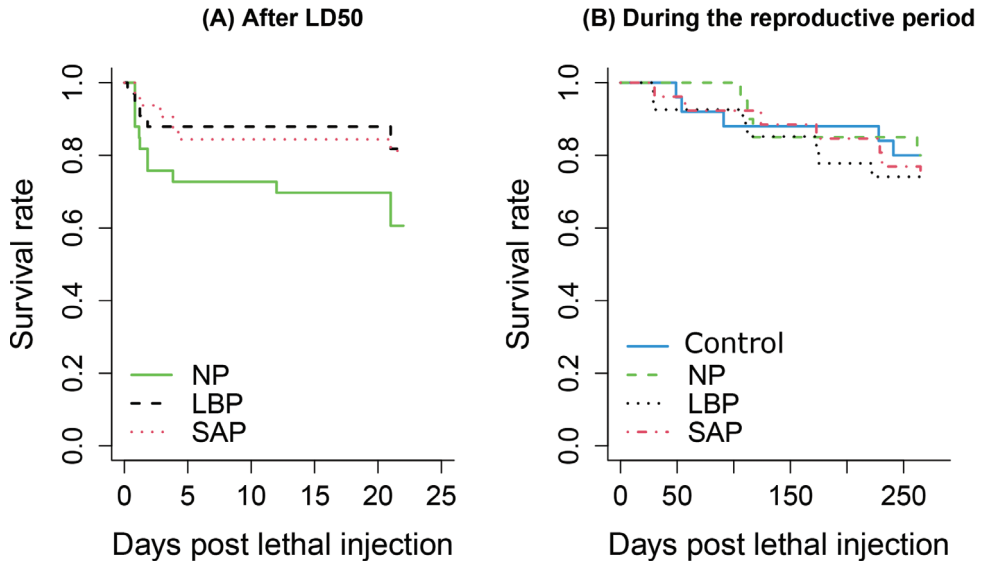


Figure 2. Survival rates **A** 22 days after the LD50 injection, and **B** during the reproductive period (ca. eight months). Abbreviations: NP: females non-primed in the priming procedure. LBP: females primed with sterile LB broth. SAP: females primed with 103 living *S. enterica*. Control: females that have never been injected. NP, LBP and SAP received the LD50 injection. Statistical results of comparisons between treatments are presented in Table S1.

weight 22% higher than that of NP females (Mean \pm SE: control = $0.15\text{g} \pm 0.008$, NP = $0.12\text{g} \pm 0.01$; estimate = 0.03, SE = 0.01, df = 82.8, $p = 0.09$; Fig. 4) and SAP females (Mean \pm SE: control = $0.15\text{g} \pm 0.008$, SAP = $0.12\text{g} \pm 0.007$, estimate = 0.02, SE = 0.01, df = 82.7, $p = 0.07$, Suppl. material 1: Fig. S1). No difference of weight was observed between the females of the other treatments (control vs. LBP: estimate = 0.01, SE = 0.01, df = 82.7, $p = 0.46$; NP vs. LBP: estimate = -0.01, SE = 0.01, df = 80.7, $p = 0.76$; control vs. SAP: estimate = -0.0007, SE = 0.0122, df = 81.0, $p = 0.99$; LB vs. SAP: estimate = 0.01, SE = 0.01, df = 81.5, $p = 0.76$).

Reproduction

First reproductive event

During the reproductive period, almost all females produced one clutch (SAP: 23/24, LBP: 20/22, NP: 22/22, control: 21/22). The probability to produce the first clutch was neither influenced by the treatment ($X^2 = 1.09$, df = 3, $p = 0.77$) nor by the weight of females before reproduction ($X^2 = 0.18$, df = 1, $p = 0.66$) or the interaction between the treatment and the weight ($X^2 = 2.61$, df = 3, $p = 0.45$). Females were able to produce the first clutch regardless of their treatment or their weight before reproduction.

The time to produce the first clutch was neither influenced by the treatment ($X^2 = 1.15$, $df = 3$, $p = 0.76$, Fig. 3) nor by the weight of females before reproduction ($X^2 = 0.49$, $df = 1$, $p = 0.48$, Fig. 3). However, the interaction between treatment and weight showed a significant effect ($X^2 = 13.32$, $df = 3$, $p = 0.003$, Fig. 3A). The time to produce the first clutch depended on the weight of females differently according to their treatment. In control females, lighter females took longer time to produce the first clutch (200 days on average) comparing to heavier control females (50 days on average; Pearson's correlation: $t = -2.34$, $df = 19$, $p = 0.03$; Fig. 3A). Conversely, in SAP females, lighter females took less time to produce the first clutch (100 days on average) comparing to heavier SAP females (200 days on average; Pearson's correlation: $t = 2.13$, $df = 21$, $p = 0.04$; Fig. 3A). In LBP and NP females, no difference in the time to produce the first clutch was observed according to their weight (LB: $t = 1.17$, $df = 18$, $p = 0.25$; NP: $t = -0.82$, $df = 17$, $p = 0.42$; Fig. 3A).

The number of offspring in the first clutch was not influenced by the female's treatment ($X^2 = 7.07$, $df = 3$, $p = 0.06$; Suppl. material 1: Fig. S2, Table S3), although control females produced an average of 134 offspring compared to an average of 90 offspring for NP and 95 offspring for SAP females (see Suppl. material 1: Fig. S2, Table S3 for Tukey's comparisons). No significant effect was observed by the body weight ($X^2 = 0.40$, $df = 1$, $p = 0.52$). However, the interaction between the body weight and the treatment influenced the number of offspring in the first clutch ($X^2 = 9.92$, $df = 3$, $p = 0.01$; Fig. 3B). Hence, the number of offspring in the first clutch depended on the female's weight differently for each treatment (Fig. 3B). Lighter SAP females

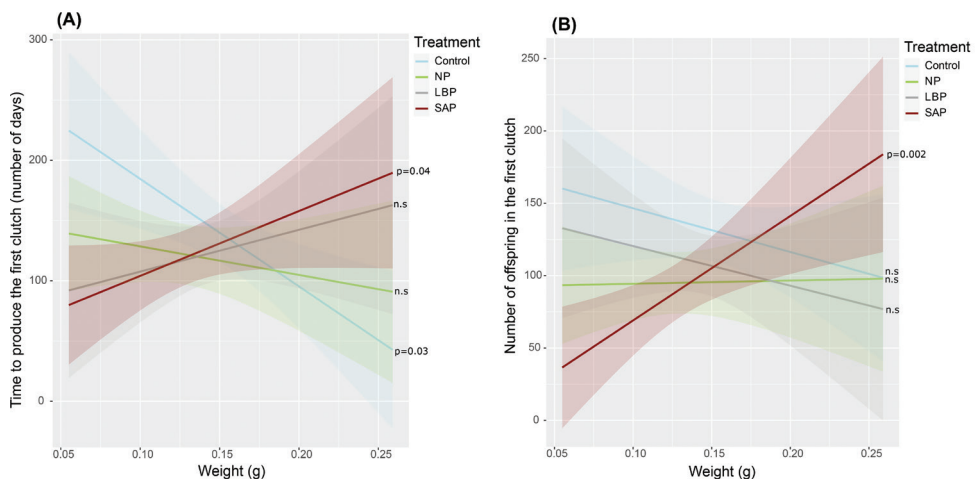


Figure 3. Interactions effects of body weight and treatment on **A** the time to produce the first clutch, and **B** the mean number of offspring in the first clutch per female. Abbreviations: control: never-injected females; NP: non-primed females; LBP: females primed with sterile LB broth, SAP: females primed with 103 living *S. enterica*. SAP, NP and LBP received the LD50 injection. P-values indicate a significant relationship between x and y axis of the considered treatment (Pearson's correlation test).

produced less offspring than heavier SAP females (Pearson's correlation test: $t = 3.50$, $df = 21$, $p = 0.002$; Fig. 3B), but the weight did not influence the number of offspring in the females of the other treatments (control: $t = -1.12$, $df = 17$, $p = 0.27$; NP: $t = 0.09$, $df = 17$, $p = 0.92$; LBP: $t = -0.73$, $df = 18$, $p = 0.47$; Fig. 3B).

Second reproductive event

Among the females which produced the first clutch, half produced a second clutch, regardless of the treatment (control: 10/21; NP: 10/19; LBP: 10/20; SAP: 11/23). Hence, the probability to produce the second clutch was not influenced by their treatment ($X^2 = 0.19$, $df = 3$, $p = 0.97$). This was neither influenced by the number of offspring in the first clutch ($X^2 = 0.20$, $df = 1$, $p = 0.64$), nor by the interaction between the treatment and the weight ($X^2 = 0.14$, $df = 3$, $p = 0.98$). However, the weight of the females influenced the probability of producing the second clutch ($X^2 = 4.60$, $df = 1$, $p = 0.03$; Fig. 4), independently of the treatment. Lighter females had higher probability (74% on average for a weight of 0.06 g) to produce a second clutch comparing to heavier ones (30% on average for a weight of 0.20 g; Fig. 4; see Suppl. material 1: Table S4 for details).

The time to produce the second clutch (after the first one) and the number of offspring in the second clutch were influenced neither by the treatment (Time: $X^2 = 3.80$, $df = 3$, $p = 0.28$; Number of offspring: $X^2 = 5.38$, $df = 3$, $p = 0.14$), nor by the weight of females (Time: $X^2 = 0.97$, $df = 1$, $p = 0.32$; Number of offspring: $X^2 = 0.54$, $df = 1$, $p = 0.45$),

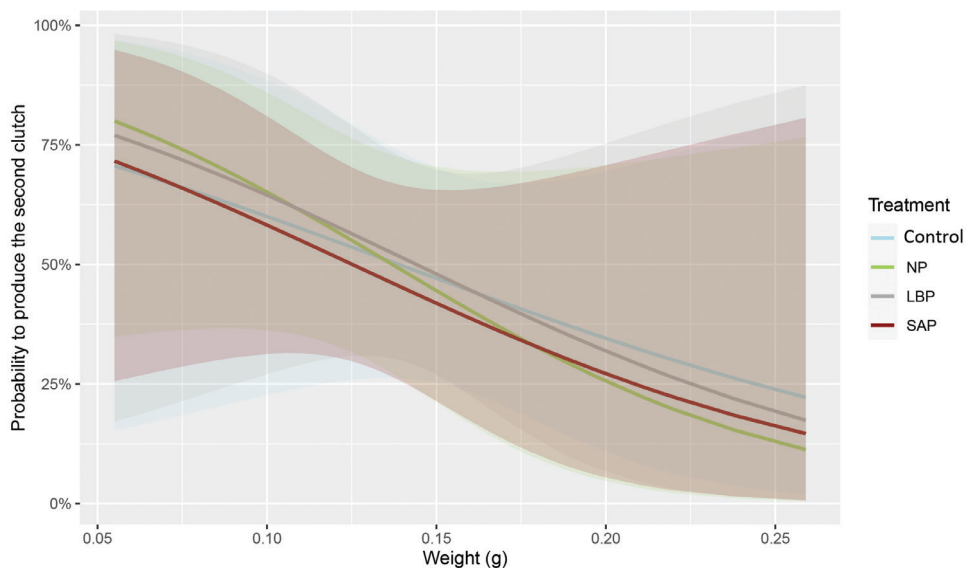


Figure 4. Probability to produce the second clutch according to female's weight and treatment. Curves were calculated using average marginal effects of the absence/presence of the second clutch (0/1) related to the weight of females. Coloured distributions represent the confident interval for each treatment (95%). Abbreviations: control: never-injected females; NP: non-primed females LBP: females primed with sterile LB broth, SAP: females primed with 103 living *S. enterica*. SAP, NP and LBP received the LD50 injection.

the number of offspring in the first clutch (Time: $X^2 = 1.68$, $df = 1$, $p = 0.19$; Number of offspring: $X^2 = 0.27$, $df = 1$, $p = 0.60$), or the interaction between the treatment and the weight ($X^2 = 2.53$, $df = 3$, $p = 0.46$). Regardless of their treatment, body weight, and cost of producing offspring in the first clutch, the females took the same time to produce the second clutch and produced a similar number of offspring in the second clutch.

Total number of offspring

The total number of offspring (first and second clutch included) was not influenced by the treatment ($X^2 = 7.46$, $df = 3$, $p = 0.058$). Even though control females produced an average of 190 offspring per female, compared to 131 offspring for SAP females (see Suppl. material 1: Fig. S3), no significant difference was observed by comparing the pairs of treatments (Suppl. material 1: Fig. S3, Table S5). The total number of offspring was neither influenced by body weight ($X^2 = 0.95$, $df = 1$, $p = 0.32$), nor by the interaction between the treatment and the body weight of females ($X^2 = 0.95$, $df = 1$, $p = 0.31$).

Haemocyte parameters and senescence biomarkers

For the haemocyte concentrations, no significant effect of any fixed factors was observed ($p > 0.05$, see Suppl. material 1: Table S6 for details). Same results were obtained

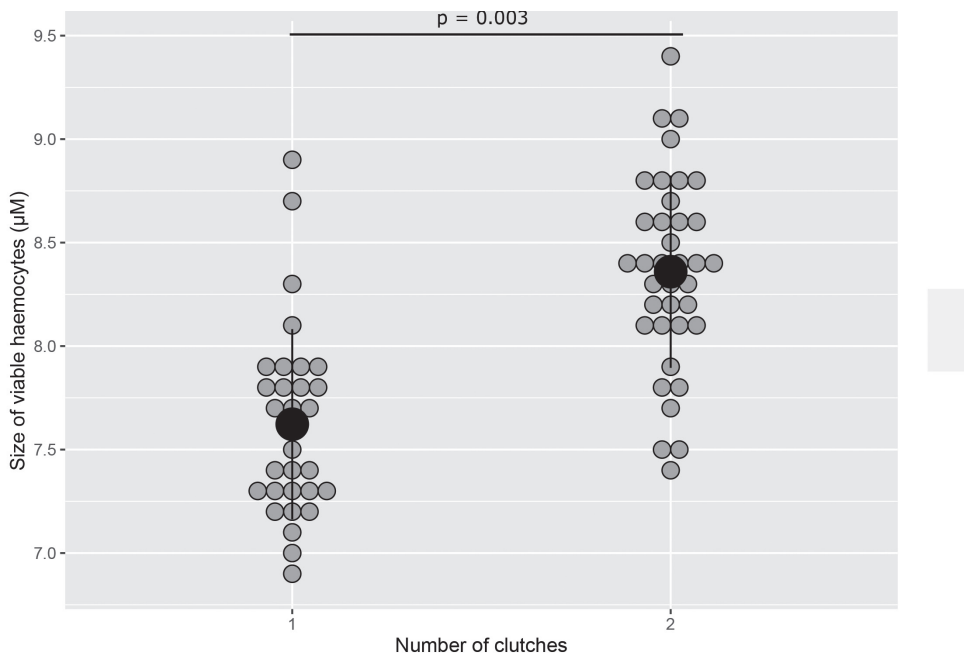


Figure 5. Size of viable haemocytes (µM) according to the number of clutch that females produced (1 or 2 clutches), all treatments combined.

for the haemocyte viabilities (Suppl. material 1: Table S6). The number of infection(s) with *S. enterica* and the following reproductive event(s) did not impact the concentration or the viability of haemocytes.

Concerning the senescence biomarkers, the size of viable haemocytes was only influenced by the number of clutches that females produced, with an increase of the cell size in the case of a second clutch production ($X^2 = 12.99$, $df = 1$, $p = 0.003$, Fig. 5). There was no influence of the other fixed factors ($p > 0.05$, see Suppl. material 1: Table S6). The β -galactosidase activity was not influenced by any of the fixed factors ($p > 0.05$, see Suppl. material 1: Table S6 for details). Whatever the treatment and the following reproductive event(s) of females, the β -galactosidase activities of females were similar.

Discussion

Our study aimed to investigate the impact of immune priming with *S. enterica* (i.e., two consecutive infections with living pathogens) on the reproductive ability and senescence biomarkers of females of *A. vulgare*. Fig. 6 illustrates our main results.

Immune priming improves the survival abilities but negatively impacts the mean body weight of females

As expected, we showed a protective effect of immune priming on female survival rates: the first encounter with *S. enterica* improves survival ability of females after the second and lethal infection, confirming previous results described in Prigot-Maurice et al. (2019, 2021). In surviving females, we showed a negative effect of immune priming on body weight of females (Fig. 6). After the LD_{50} , females that received one or two infection(s) with *S. enterica* (NP or SAP) tended to be lighter than females that were never injected (control). Because *S. enterica* infection(s) is expected to induce metabolic costs related to immune functions like other pathogenic infections in invertebrates (Schwenke et al. 2016), the lower body weight of females could be explained by two non-exclusive hypotheses. It could either indicate (1) a decrease of the energetic resources of females (e.g., fat body), or (2) a slowdown in growth at the expense of investment in immunity, since body weight and size are closely related in *A. vulgare* (Antoľ and Czarnoleski 2018; Durand et al. 2018; Schwenke et al. 2019). Interestingly, the injection of sterile LB Broth did not impact body weight of females, while these females have also been exposed to the second infection with living *S. enterica*. This result does not support the findings of other studies showing that individuals injected with non-pathogenic immunostimulants (e.g., LPS) became lighter as well (*T. molitor*, Moret 2006; *Hemedeina crassidens* (Blanchard, 1851), Kelly 2011). In *A. vulgare*, the first immune stimulation without pathogens (LBP treatment) appears to prevent the energetic cost of the second infection with *S. enterica*. Similar results have been observed in *Cambarus clarkia* (Girard, 1852), for which individuals previously injected with live attenuated *Salmonella* do not show variation in body weight in the two months following infection (Ning et al. 2009).

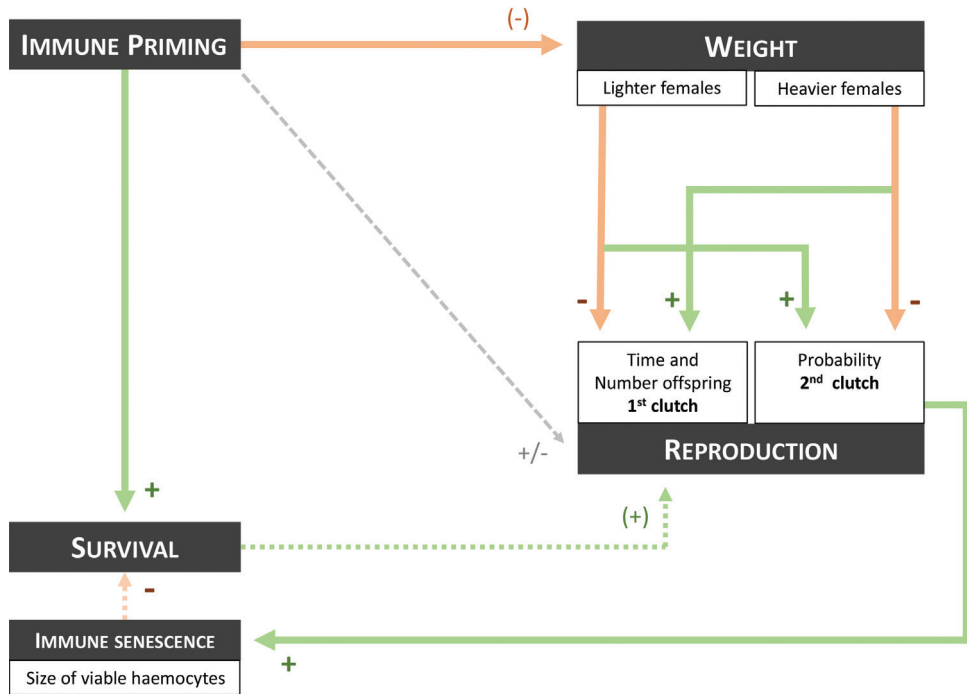


Figure 6. Summary diagram of the results. Orange arrows indicate a negative effect. Green arrows indicate a positive effect. If the corresponding sign is in brackets, the effect is a trend ($0.05 < p < 0.10$). +/- indicates no general effect.

No evidence of immune priming costs on the reproductive abilities of double infected females

Since energetic investment in immunity often reduces available energy to produce offspring, the negative impact of immune responses on reproductive ability is widely observed across invertebrate species (Schwenke et al. 2016). While we hypothesised that the persistent infection of *S. enterica* during immune priming in *A. vulgare* would also negatively impact its reproductive abilities, we didn't find any statistical evidence of lower probability to produce clutch(es) or lower number of offspring in females experiencing two consecutive infections with living *S. enterica* (SAP). All females infected (NP, LBP, and SAP) were as likely to reproduce as females that were never infected (control). In addition, the females that mounted immune priming (SAP) have similar survival rates during the reproductive period to females that have received only one (NP, LBP) or no injection (control females; Fig. 2B). Hence, females of *A. vulgare* maintain high survival rates with no apparent reproductive cost, while cellular changes are sustained for at least seven days, and *S. enterica* persists in the haemolymph of the females for fifteen days after the infections (Prigot-Maurice et al. 2019).

Most studies that investigated the costs of mounting immune priming showed a negative impact on reproduction (Schwenke et al. 2019). However, from an evolutionary point of view, a biological process inducing higher costs than benefits would be counter-selected. This counter-selection would be particularly strong when the biological process reduces the reproductive ability of individuals because it also reduces the possibility to transmit this process to the next generation. From this statement, it appears that immune priming should be selected during evolution if it does not induce a high cost (Moret 2003). Very few studies have shown absence of cost when investigating the evolutionary outcome of immune priming or transgenerational immune priming (Tetreau et al. 2019). In females of *T. molitor*, for instance, infection with *A. globiformis* or *B. thuringiensis* does not impact the number of eggs but increases the hatching rate of these eggs (Dhinaut et al. 2018). In the study of Bordoni et al. (2018), immune primed queen ants of *Crematogaster scutellaris* (Olivier, 1792) produced as many worker offspring as control queens. In addition, these ant workers displayed higher survival rates compared to workers derived from non-primed queens (Bordoni et al. 2018). By demonstrating that immune priming is not always associated with reproductive costs, these studies give important clues about the adaptive nature of immune priming. In our study, the absence of cost on immediate reproduction in female of *A. vulgare* expressing immune priming agrees with this but raises the question of how this immune process could be exempted from reproductive costs.

If the energetic resources of individuals are limited and trade-offs are inevitable between reproduction and immune response to infection (Schwenke et al. 2016), natural selection should favour the most optimal immune responses facing these allocations (Rauw 2012). To explain the reduction of reproductive costs following infection, we supposed that immune priming relies on increased tolerance against *S. enterica* rather than increased resistance, which would require much more energy at the expense of other biological functions (Zuk and Stoehr 2002; Rauw 2012). However, as stated by Tetreau et al. (2019), “absence of evidence does not always mean evidence of absence”. Indeed, no evidence of reproductive cost induced by immune priming does not mean there is no cost at all. It could result from the lack of statistical power and/or from methodological bias, where the costs are associated with other life-history traits that were not measured during the experiment. One important trait to consider is the total lifespan of females (i.e., their abilities to reproduce in later reproductive season), but more importantly, the life-history traits of the offspring of immune primed females. Immune priming is often associated with costs in the following generations, such as longer developmental time, reduced fecundity or reduced immune functions of offspring (for review see Tetreau et al. 2019). In *Crassostrea gigas* (Thunberg, 1793), offspring from immune primed mothers take longer to develop, which delays their sexual maturity compared to offspring from non-primed mothers (Robinson and Green 2020). In the present study, the total number of offspring of females is not altered by double infections of *S. enterica*, but this does not prove that the fitness of these offspring is not negatively impacted. Hence, to conclude about the adaptive nature of immune priming in *A. vulgare*, it is required to look at the reproductive, growth and survival abilities against pathogens of offspring derived from females that have

established immune priming during their lifetime. If the trans-generational benefits of immune priming in offspring are higher than costs, then immune priming in this species should be adaptive in an evolutionary sense.

Indirect effect of immune priming on the first clutch production through the body weight of females

Even though we observed no evident cost on reproduction in females receiving the double infection of *S. enterica*, the treatment of females indirectly influences their reproductive strategies through body weight. In never-injected (control) females, the lighter ones took a longer time (200 days on average) to produce the first clutch than heavier ones (50 days on average; Fig. 3A). We suggest that this difference in body weight in control females is the result of a strategy to optimise reproduction in non-stressful conditions. Producing clutch is an expensive event in *A. vulgare*, because the production of the marsupium requires considerable energy by the female (Surbida and Wright 2001; Antoł and Czarnoleski 2018). However, the size of the marsupium is proportional to the size of the female, making the largest females the ones that normally produce more offspring by clutch (Antoł and Czarnoleski 2018). Because larger females *A. vulgare* have a higher fecundity (Waller and Verdi 2016; Durand et al. 2018), it is in the interest of females to grow before the reproductive event (under non-stressful conditions), also supported by the indeterminate growth ability of terrestrial isopods (Antoł and Czarnoleski 2018). By investing energy in their growth, thus delaying the production of the first clutch, lighter control females would indirectly invest in their reproduction by preparing the optimal physical conditions to produce as many offspring as possible in the following clutch (Lawlor 1976). We observed that lighter control females produced as many offspring in the first clutch as heavier control females (Fig. 3B), which supports the idea of an energetic investment in growth before reproduction. This result is in line with Warburg (2011), who demonstrated no relationship between the original body weight of females and the number of offspring in four different species of terrestrial isopods maintained in non-stressful conditions.

Otherwise, immune priming of double-infected females also induces different effects on the production of the first clutch according to body weight (Fig. 6). Indeed, two different strategies of reproduction were observed in females that experienced two consecutive infections with *S. enterica* (Figs 3, 6). The lighter SAP females took less time to produce the first clutch (100 days on average) but produced less offspring in this first clutch (50 offspring on average; Fig. 3) compared to heavier SAP females. In contrast, the heavier SAP females took more time to produce the first clutch (200 days on average), but it contained more offspring (150 offspring on average; Fig. 3).

From our point of view, these reproductive patterns in SAP females result from an alteration in energetic resource allocation. During a stressful event occurring in the lifetime of an organism, like an infection, it could opt for the investment of its remaining energy in reproduction, at the expense of growth, in order to maximise fitness before dying (Creighton et al. 2009). Hence, when females of *A. vulgare* have used a lot of energy in immunity against the double infection with *S. enterica* (i.e., are lighter), they

probably have less remaining energy to invest in growth and/or to produce numerous offspring. The lower number of offspring in the first clutch of the lighter SAP females could thus be the consequence of constraints by marsupium size (i.e., it might also be smaller) and/or the reduction of available energy for numerous offspring (if marsupium size is similar with that of heavier females). For both possible cases, the lighter SAP females produced a lower number of offspring in the first clutch compared to the heavier ones, which probably requires less incubation time in the marsupium, thus reducing the number of days to produce the first clutch (Antol and Czarnoleski 2018). For the heavier SAP females, we assume that the costs of immunity during the infections of *S. enterica* were lower than those of lighter SAP females. As a result, heavier SAP females may have more energy for growth and/or to produce numerous offspring that take more time to complete their embryogenesis (Antol and Czarnoleski 2018). To test this hypothesis, it would be possible to supplement the food of SAP females with metabolites. For instance, aquatic crustaceans fed with Lysine, Arginine, or Threonine-rich diet display higher body weight and reproductive ability (for review: Huang et al. 2020). If SAP females of *A. vulgare* fed with supplemented diet gain in body weight and increase their reproductive abilities compared to poor-diet SAP females, then the lower reproductive ability of poor-diet SAP females will demonstrate the energetic allocation toward immunity to the detriment of reproduction (and maybe growth) in these lighter females.

These two different strategies illustrate a plasticity in resource allocation following two infections with *S. enterica* that depends on the investment of each female in the different physiological functions, namely somatic maintenance (including response to pathogens and/or growth) and reproduction. However, the total number of offspring per female (first and second clutches included) was influenced neither by the number of infections nor by body weight or the interaction between these parameters. Hence, whatever the allocation strategy of energetic resources in the first clutch in SAP females, the lighter of them mobilise enough energy to finally produce as many offspring as the heavier SAP females. This lack of effect seems explained by the second reproductive event.

Heavier females have reduced probability to produce a second clutch, regardless of the number of infections

Concerning the second reproductive event, the probability of producing the second clutch only depended on the body weight of the females: the heavier the females are, the less likely they are to produce a second clutch (ca. 25%) compared to lighter females (ca. 75%). We suppose that the investment in the first clutch by heavier females is more expensive than for those lighter ones, regardless of treatment. As we have already stated, the costs of producing one marsupium are high, and positively correlated to female size (Dangerfield and Telford 1995; Lardies et al. 2004; Antol and Czarnoleski 2018). For instance, Antol and Czarnoleski (2018) demonstrated that clutch size and clutch mass increased with female body mass in the terrestrial isopod *Porcellio scaber* (Latreille, 1804). In *Porcellio laevis* (Latreille, 1804), the heavier females have higher metabolic rates during eggs incubation comparing to lighter ones (Lardies et al. 2004). Hence, we suppose that the heavier females may not have enough remaining energy (after the first clutch) to produce a second marsupium in

the time course of our experiment. Here, we decided to stop the experiment at the end of the first reproductive season (ca. eight months after infections). The probability of producing a second clutch must be considered for this period. It does not mean that heavier females will not be able to produce other clutches in the second reproductive season. Several studies on crustaceans demonstrate that basal metabolic rates (estimated by protein synthesis and oxygen uptake) increase with individual body weight (Houlihan et al. 1990; Whiteley et al. 1996, 2001), indicating the allocation of energetic resources in somatic maintenance (Whiteley and Faulkner 2005). To confirm our previous hypothesis, the oxygen consumption and the rate of proteins synthesis could be used to compare the energetic costs of producing the first clutch between the heavier and the lighter females of *A. vulgare*.

Nevertheless, this result raises questions about the intrinsic factors that cause the production of one or two clutch(es) in females of *A. vulgare* depending on body weight, regardless of treatment. The first assumption about the lower probability to produce two clutches by heavier females is related to the production of better-quality offspring. According to theoretical predictions, females producing a single clutch should provide higher rates of care to their offspring than those producing several clutches (Tallamy and Brown 1999; Gilbert and Manica 2010; Meunier et al. 2012). In *Forficula auricularia* (Linnaeus, 1758) for example, females providing more pronounced maternal care to the offspring in the first clutch are less likely to reproduce later (Koch and Meunier 2014). The quality of offspring is often measured through their size/weight at birth because it would improve survival abilities of offspring (Brody and Lawlor 1984; Dangerfield 1997), especially in species where the ability to grow is indeterminate, like in terrestrial isopods (Hubbel 1971; Lawlor 1976; Dangerfield and Telford 1995). In nine different species of isopods, there is evidence of positive correlation between female size and offspring size, thus potentially offspring quality (Antol and Czarnoleski 2018). In *A. vulgare*, females maintained in a resource-poor environment produce larger offspring compared to females maintained in usual environment (Brody and Lawlor 1984). However, a greater investment of parents in offspring size, does not always increase the fitness of their offspring (Depeux et al. 2020a). In *A. vulgare*, older parents produce larger and more numerous offspring than younger ones, but the offspring of these older parents have lower survival abilities and are unable to reproduce (Depeux et al. 2020a). Complementary experiments are thus needed to evaluate the quality of offspring of females producing only one clutch, by following their survival and reproduction in the adult stage (Depeux et al. 2020a).

The second assumption concerning the probability to produce one or two clutches refers to the environmental and physiological conditions of the females before reproduction. By observing the reproductive phenology in four different species of terrestrial isopods, Dangerfield and Telford (1995) postulated that: “the tactic of repeated reproduction, with a relatively conservative allocation of resources to each reproductive event to enhance survival probabilities, would be favoured, particularly in an environment where juvenile mortality and the chance of complete brood failure is high”. The likelihood of clutch failure and offspring mortality could be sensed by females when the environmental and physiological conditions vary, such as the increase/decrease of temperature, food intake (i.e., energetic resources), or pathogenic infections (Brody and Lawlor 1984; Dangerfield and Hassall 1992; Dangerfield and Telford 1995; Hassall et al. 2005). In the case

of pathogenic infections in invertebrates, for instance, level and frequency of pathogens and danger-associated molecular patterns may indicate the pathogen's abundance and persistence in the environment where the offspring will be produced. Hence, the lower weight of females, maintained in infectious conditions or not, could also be a signal to promote individual fitness, as in many species (Mousseau 1998; Kim et al. 2017). If the lighter females have less available energy for their offspring for a given time or have smaller marsupia that constraint the oviposition of numerous eggs, then they may counterbalance the lower number of offspring in the first clutch by producing a second clutch as soon as they acquire enough energy to do so. Our results about the lighter females experiencing two consecutive infections with living *S. enterica* (i.e., that sense environmental and physiological stressful conditions) confirm this assumption. With their higher probability to produce a second clutch compared to heavier females, they produce a similar total number of offspring as the heavier females that, however, produce only one clutch.

While immune priming neither impacts the haemocyte parameters nor the senescence patterns after reproduction, producing two clutches increases the cellular senescence of females

For the senescence biomarkers, we only observed an effect of the investment in reproduction on the size of the viable haemocytes: regardless of treatment, females that have produced a second clutch had larger viable haemocytes than those of females that produced only one clutch. The size of haemocytes in *A. vulgare* increases with the age of individuals, making this morphometric trait a biomarker of senescence (Depeux et al. 2020b). Here, for an identical age of females, the larger size of haemocytes could therefore show premature senescence in females that produced two clutches, suggesting a decrease in total lifetime. This result could illustrate a trade-off between the energy allocated to reproduction or somatic maintenance. To confirm this cellular senescence state, other biomarkers of senescence should be analysed, as the TERT gene expression (Depeux et al. 2020b), or the telomere length for instance. However, the higher viable cell size could also indicate different proportion of haemocyte types. In *A. vulgare*, hyalin and semi-granular haemocytes are taller than granular haemocytes (Chevalier et al. 2011). Hence, females producing two clutches could have higher proportion of hyalin and semi-granular haemocyte types comparing to females producing only one clutch, but further experiments (with cytometric analysis for instance), are also needed to confirm this hypothesis.

Finally, we observed no effect of the treatment or the reproductive event(s) on the haemocyte parameters of females (concentration and viability). We conclude that several months after *S. enterica* infection(s), immune cell production is no longer impacted by infection(s) (Prigot-Maurice et al. 2019), nor by the following reproductive event(s). However, these immune parameters only reflect the quantity and basal viability of haemocytes of the females. This does not mean that the immune activities of these haemocytes (i.e., the immunocompetence of females against a future threat) is not impacted by infectious and reproductive events (Lawniczak et al. 2007), since the females that have produced two clutches displayed higher cellular senescence pattern through the size of their viable haemocytes.

Conclusions

Our study aimed to investigate the impact of immune priming with *S. enterica* on life-history traits and senescence biomarkers in *A. vulgare*. While current studies in various species show negative effects of immune priming, we only found an indirect effect of immune priming by body weight of females that could indirectly impact reproduction. However, we observed no strong effects of consecutive infections with *S. enterica* in the reproductive ability of female. Even though the absence of evidence for cost(s) does not mean that there is no cost at all, the fact that only a few studies reporting the absence of costs of immune priming or transgenerational immune priming could be explained by the difficulty to publish non-significant results. The publishing bias towards significant results of immune priming costs can change our view of evolutionary implications (Tetreau et al. 2019). Hence, our study tends to highlight the adaptive potential of immune priming in *A. vulgare* during evolution. Because *A. vulgare* is a detritivore and gregarious organism living for several years in moist environments particularly rich in microbial density and diversity (Warburg et al. 1984; Ranjard and Richaume 2001; Zimmer 2002; Broly et al. 2013; Bredon et al. 2020), the decreasing cost of immune priming on reproduction in an evolutionary scale for this species suggests its important role. Overall, our results tend to show that immune priming is not always associated with significant reproductive costs, even if the underlying mechanism is a sustained immune response lasting from the first to the second infection (Prigot-Maurice et al. 2019). Increasing studies on this subject will shed light on evolutionary mechanisms that have favoured immune priming over time.

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Supplementary material I

Tables S1–S4, Figures S1–S3

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Data type: Pdf file.

Explanation note: **Table S1.** Pairwise comparisons (Tukey adjustment) of survival rates according to females' treatments (NP: non-primed females; LBP: females primed with sterile LB broth; SAP: females primed with 103 *S. enterica* in the priming procedure; Control: never-injected females). **Figure S1.** Body weight of females before reproduction according to their priming treatment. NI: never-injected, control females; NP: non-primed females; LBP: females primed with sterile LB broth, SAP: females primed with 103 living *S. enterica*. NP, LBP and SAP females received the LD50 injection. Mean \pm SE: Control = $0.15\text{g} \pm 0.008$, NP = $0.12\text{g} \pm 0.01$, LBP = $0.14\text{g} \pm 0.007$, SAP = $0.12\text{g} \pm 0.007$). **Table S2.** Pairwise comparisons (Tukey adjustment) of body weight of females before reproduction, according to the treatments (NP: non-primed females; LBP: females primed with sterile LB Broth; SAP: females primed with 103 *S. enterica* during the priming procedure; Control: never-injected females). **Figure S2.** Number of offspring in the first clutch according to the females' treatment. NI: never-injected, control females; NP: non-primed females; LBP: females primed with sterile LB broth, SAP: females primed with 103 living *S. enterica*. NP, LBP and SAP females received the LD50 injection. **Table S3.** Pairwise comparisons (Tukey adjustment) of the number of offspring in the first clutch according to females' treatments (NP: non-primed females; LBP: females primed with sterile LB Broth; SAP: females primed with 103 *S. enterica* during the priming procedure; Control: never-injected females). **Figure S3.** Total number of offspring (first and second clutches included) according to the females' treatment. NI: never-injected, control females; NP: non-primed females; LBP: females primed with sterile LB broth, SAP: females primed with 103 living *S. enterica*. NP, LBP and SAP females received the LD50 injection. **Table S4.** Average marginal effects of the interaction between the probability to produce a second clutch and the body weight of females by treatment (NP: non-primed, Control females; LBP: females primed with sterile LB Broth; SAP: females primed with 103 *S. enterica* in the priming procedure; Control: never-injected females). **Table S5.** Pairwise comparisons (Tukey adjustment) of the total number of offspring according to females' treatments (NP: non-primed, Control females; LBP: females primed with sterile LB Broth; SAP: females primed with 103 *S. enterica* in the priming procedure; Control: never-injected females). **Table S6.** Statistical results of haemocyte parameters and senescence biomarkers analysis (generalized linear mixed effect models).

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Formation and remodelling of septate junctions in the epidermis of isopod *Porcellio scaber* during development

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Abstract

Septate junctions (SJs) perform an occluding function in invertebrate epithelia and consist of parallel septa extending across the intercellular space between neighbouring cells. In addition, they are required for several morphogenetic processes in arthropods. The biogenesis of SJs during development is inadequately studied and it was characterised in detail only for various epithelia of *Drosophila melanogaster*. This paper provides a detailed analysis of the ultrastructural differentiation of SJs in the epidermis of the terrestrial isopod *Porcellio scaber* during embryonic and postembryonic development. In this study, mid-stage embryo S13 was the earliest stage in which single septa were observed basally to the adherens junction (AJ). Differentiation of SJs during further development includes gradual elongation of septa arrays and formation of continuous arrays of septa. The enlargement of SJs in the epidermis is most pronounced at the transition from embryonic to postembryonic development and after the release of mancae from the marsupium. SJs of postmarsupial mancae are similar to those of adults, but are not yet as extensive. Comparison of the differentiation of SJs in the epidermis and hindgut of *P. scaber*, reveals a similar sequence of events. In addition, remodelling of SJs was observed in the epidermis of late marsupial mancae, the stage of cuticle renewal. Common features of SJs' biogenesis in *P. scaber* and *D. melanogaster* ectodermal epithelia are indicated.

Keywords

Crustacea, embryo, epithelia, junctional complex, morphogenesis, ultrastructure

Introduction

The epidermis functions as a protective barrier, as well as a sensory interface between an organism and the outer environment. Its apical surface faces the exterior and in arthropods is covered by a cuticle, which provides additional protection (Neville 1984; Compère et al. 2004; Dillaman et al. 2013). The epidermis of arthropods is a monolayered epithelium, whose cells are connected by adherens junctions (AJ) and septate junctions (SJ) (Tepass and Hartenstein 1994; Izumi and Furuse 2014; Jonusaite et al. 2015). AJs mainly provide mechanical stability to the tissue and SJs function as an occlusive barrier to paracellular transport and are thus involved in the regulation of transepithelial transport. In arthropod epithelia and in invertebrate epithelia in general, the AJs are located subapically. They are discernible as two electron-dense plaques on the cytoplasmic sides of neighbouring lateral cell membranes, separated by an intercellular space of constant width, which is filled with less dense homogenous material. In vertebrates, AJs are located basally to tight junctions, which occlude the space between neighbouring cells. In invertebrate epithelia, the occluding function is performed by SJs, which were first reported in two species of cnidarians by Wood (1959), and termed septate desmosomes. SJs consist of parallel septa which surround the cell circumferentially (Fristrom 1988; Tepass and Hartenstein 1994; Tepass et al. 2001; Izumi and Furuse 2014; Jonusaite et al. 2015). In sections that are perpendicular to the septa, their “ladder-like” ultrastructure is clearly discernible, as electron dense septa span an intercellular space of constant width. Distinct morphological variants of SJs have been described in different invertebrate species and in different epithelia (Izumi and Furuse 2014; Jonusaite et al. 2015). In arthropod epithelia, smooth and pleated SJs have been characterised (Flower and Filshie 1975; Noirot-Timotheé et al. 1978; Tepass and Hartenstein 1994; Izumi and Furuse 2014; Jonusaite et al. 2015). Smooth SJs are present in endodermally derived epithelia and pleated SJs in ectodermally derived epithelia, including the epidermis and hindgut. Pleated SJs are located basally to the AJs. Structurally similar pleated SJs are a hallmark of mollusc epithelia, where they have been shown to differ in permeability in accordance with the physiological function of different epithelia. This has reinforced the concept that they have a versatile and dynamic role (Jonusaite et al. 2015).

Functions beyond the role of SJs as a diffusion barrier have been reported and involvement of SJ proteins in regulation of morphogenesis and in signal transduction pathways has been demonstrated (Lamb et al. 1998; Luschnig et al. 2006; Hall and Ward 2016; Lim et al. 2019; Rouka et al. 2020). Different SJ proteins have been shown to be essential for several morphogenetic processes (Hall and Ward 2016; Rouka et al. 2020). They are required for hindgut morphogenesis (Wells et al. 2013), morphogenesis of trachea (Bätz et al. 2014) and salivary glands (Hall and Ward 2016). The role of SJ proteins in cell shape changes during salivary glands morphogenesis has been suggested. Interestingly, many SJ protein mutants show defects in the architecture of cuticular structures. Delamination between epicuticle and procuticle was reported

(Lamb et al. 1998) and irregular organisation in the tracheal taenidia (Wu et al. 2004) was observed. Data on the structural characteristics of the formation of intercellular junctions during development are very scarce and are mainly limited to studies of the model organism *Drosophila melanogaster* (Tepass and Hartenstein 1994; Genova and Fehon 2003; Tiklová et al. 2010). Several studies have documented the key role of SJs for normal development of *Drosophila* (Tepass et al. 2001). Growing evidence also supports the view that SJ proteins are involved in a spectrum of developmental events in which their function is probably independent of their involvement in occluding establishment of SJs (Rice et al. 2021).

To advance the understanding of the biogenesis and function of SJs it is necessary to analyse the ultrastructural differentiation of SJs in different species and to compare the timing of major events in their biogenesis with the steps in the embryonic and postembryonic development of the organism. It is also advantageous to evaluate the biogenesis of SJs in different organs of the same species to identify common and/or tissue specific principles of the formation and function of SJs. In arthropods, the isopod crustacean *Porcellio scaber* Latreille, 1804 is a suitable species to address this issue as its embryonic and postembryonic development is well characterised by morphological staging systems (Wolff 2009; Milatović et al. 2010). In addition, data on morphogenesis of the digestive system and the epidermis are available (Štrus et al. 2008; Mrak et al. 2014; Bogataj et al. 2019). Embryonic development and the first stages of postembryonic development of *P. scaber* take place in the aqueous environment of the marsupium, a brood pouch on the ventral side of the female. The early-stage embryos comprise stages S1-S5, mid-stage embryos stages S6-S15, and after hatching from the chorion, the outer egg envelope, embryos are termed late-stage embryos, through stages S16-S19. After release from the inner egg envelope, the vitelline membrane, marsupial mancae, including early-stage, mid-stage, and late-stage marsupial mancae develop within the marsupium for up to ten additional days (Milatović et al. 2010; Mrak et al. 2012). During this early postembryonic development, the epidermis forms a new cuticle (Mrak et al. 2014). Postembryonic development proceeds after release from the marsupium as postmarsupial mancae stages, which are adapted to the external terrestrial environment (Tomescu and Craciun 1987). To the best of our knowledge, the ultrastructural differentiation of cell junctions in the epidermis of crustaceans has not been characterised to date. A recent study by Bogataj et al. (2019) provides a detailed ultrastructural analysis of the differentiation of SJs in the hindgut epithelium during development of *P. scaber*.

In this study we characterise the ultrastructural differentiation of SJs in the epidermal epithelium of *P. scaber* during embryonic and postembryonic development, based on transmission electron microscopy imaging and measurements of SJs' structural characteristics. Our results are evaluated and discussed with respect to data on differentiation of SJs in the well-studied model organism *Drosophila melanogaster* and with respect to SJ differentiation in the hindgut epithelium of *P. scaber*, aiming to unravel common features in the biogenesis of pleated SJs.

Materials and methods

Specimens of *Porcellio scaber*

Specimens of *P. scaber* Latreille, 1804 (Crustacea: Isopoda) were collected in Slovenia and placed in a glass terrarium with soil and leaf litter. Animals were maintained and bred at a constant temperature of 25 °C, high humidity and a 12 h light/dark cycle. Adult animals without a marsupium and without external signs of moulting were included in the analysis (Zidar et al. 1998). Embryos and marsupial mancae were isolated from the marsupia of gravid females. Embryonic developmental stages were characterised according to morphological characteristics defined in the developmental staging system established by Milatović et al. (2010). Among 19 embryonic stages, the following stages were analysed in this study: five stages of mid-stage embryos (S10, S12, S13, S14, and S15) and two stages of late embryos (S16 and S18). The marsupial mancae stages were defined by morphological characteristics described in Mrak et al. (2012) as early-, mid-, and late-stage marsupial mancae. Postmarsupial mancae were collected three days or two weeks after release from the marsupium of the females which were reared individually in moist petri dishes. The images of specimens in different developmental stages were recorded with a MZFL III stereomicroscope (Leica) equipped with a Leica DFC425 C digital camera, using LAS V4.0 software.

Sample preparation and imaging by light microscopy and transmission electron microscopy

Adult animals were anaesthetised with diethyl ether before dissection. Tergites were isolated and cut along the median plane, fixed and decalcified overnight in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde and 2.5% ethylenediaminetetraacetic acid (EDTA) in 0.1M HEPES buffer (pH 7.2). Intact embryos and mancae were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 2 h and stored in the fixative at 4 °C for several days, needed to collect samples of different developmental stages. Egg envelopes surrounding the embryos were perforated with a thin needle or completely removed before fixation. Subsequent to fixation, all samples were rinsed with the same buffer that was used in the fixative and then post-fixed for 2 h in 1% osmium tetroxide (OsO₄). After rinsing with buffer, the specimens were dehydrated in ethanol, graded from 50% to 100%, transferred to pure acetone and finally infiltrated and embedded in Agar 100 epoxy resin. Prior to embedment, the surface of each manca was carefully perforated with a thin needle to improve infiltration of the resin. Resin polymerisation was performed at 60 °C for at least 24 h.

Semithin sections (0.5 µm) of the samples for light microscopy and ultrathin sections (~70 nm) for transmission electron microscopy were cut with a glass and a diamond knife respectively, using a Reichert Ultracut S ultramicrotome (Leica). The semithin sections were stained with Azure II – Methylene Blue (Richardson stain), dried and mounted in Ultrakitt (J.T. Baker) then inspected with an Axioscope Opton (Zeiss) light microscope. Micrographs of tergites and the dorsal body surface of embryos and

mancae were obtained with a Leica DFC290HD digital camera using LAS V4.0 software. Ultrathin sections were contrasted for 10 min with uranyl acetate and for 5 min with lead citrate. They were analysed and imaged with a CM100 (Philips) transmission electron microscope, equipped with an Orius SC200 digital camera (Gatan) and Digital Micrograph software. Electron micrographs of tergites and the dorsal body surface of embryos and mancae were acquired and analysed.

Measurements of structural characteristics of adherens and septate junctions

Measurements of selected structural characteristics of cell junctions were performed using ImageJ/Fiji software on electron micrographs obtained in seven embryos (three mid-stage embryos S13 and four late-stage embryos S16), seven marsupial mancae (two early-, two mid-, three late-stage), in four postmarsupial mancae (postmarsupial mancae 3 or 14 days after release from marsupium, two of each) and in three adult animals. The following characteristics of the SJs and AJs were measured: (i) the length of a continuous array of septa, (ii) the spacing between consecutive septa in an array, (iii) the thickness of septa, (iv), the width of intercellular space in the SJ region, (v) the width of intercellular space in the AJ region, (vi) the distance of the AJ from the apical membrane and (vii) the length of the AJ (Fig. 1). In the measurement of the length of a continuous array of septa, the longest array of consecutive septa of each SJ was included, while subsequent or previous arrays that were shorter than the measured array, were not included. Measurements were carried out as follows. The length of a continuous array of septa was measured with the “segmented line” tool in the ImageJ/Fiji software, as shown in Fig. 1A. The spacing between consecutive septa in an array (Fig. 1B) and the thickness of septa (Fig. 1C) were measured with the “straight line” tool. The width of intercellular space was measured using the “straight line” tool as shown in Fig. 1D for SJs and as shown in Fig. 1E for AJs. The distance of the AJ from the apical membrane was measured with the “segmented line” tool in the Fiji software by lining the lateral membrane from the apical margin of the AJ to the apical membrane (Fig. 1F). The length of the AJ was measured using the “segmented line” tool (Fig. 1G).

To determine the statistically significant differences of junctions’ structural characteristics between groups the Kruskal-Wallis test was performed, followed by Mann-Whitney pairwise test with Bonferroni correction. In addition, to determine the statistically significant differences in the width of the intercellular space in the region of AJs compared to SJs, two sample Mann-Whitney tests were performed. Due to small sample sizes nonparametric tests were applied. All statistical tests were performed using PAST v4.03 software (Hammer et al. 2001; <https://www.nhm.uio.no/english/research/infrastructure/past/>). Data visualisation was performed by box-and-whiskers plots, generated using BoxPlotR, a web-tool for generation of box plots (Spitzer et al. 2014; <http://shiny.chemgrid.org/boxplotr/>). The edges of the box are the first (Q1) and the third (Q3) quartile, while the second quartile (Q2), the median, is represented by the line in the box. The whiskers represent the lowest and highest data within 1.5*IQR from the first and third quartile, respectively. Individual measurements are represented with dots.

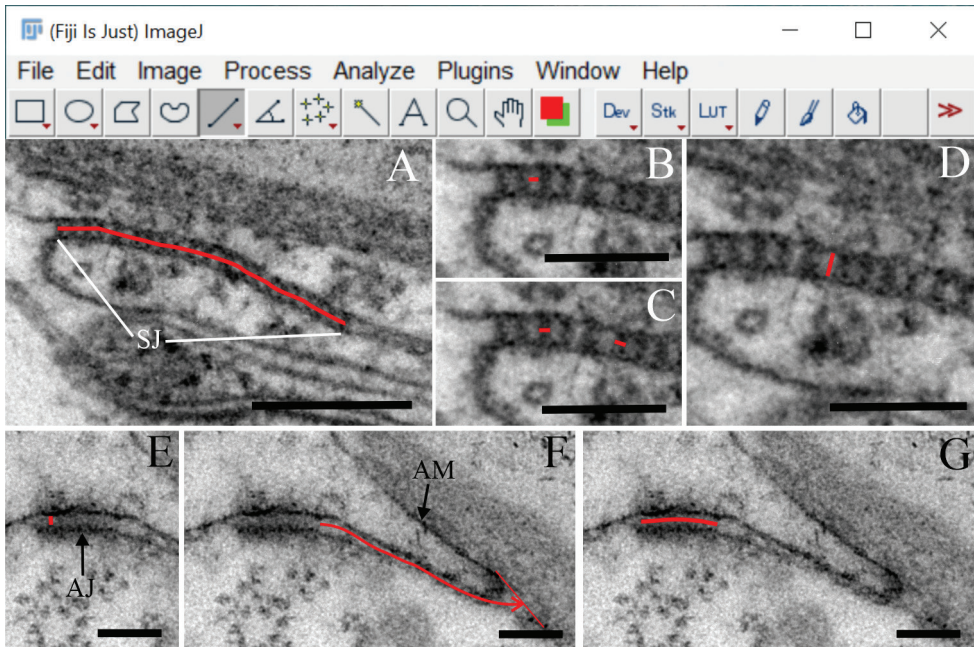


Figure 1. Measurements in ImageJ/Fiji **A–D** measurements of SJ characteristics **A** the length of a continuous array of septa was measured using the “segmented line” tool **B** the spacing between consecutive septa in an array is indicated **C** the thickness of septa is labelled **D** the width of intercellular space in the SJ region **E–G** measurements of AJs **E** the width of intercellular space in the AJ region **F** measurement of the distance of AJ from the apical membrane (red arrow) **G** measurement of the AJ length. Abbreviations: AJ: adherens junction; AM: apical membrane; SJ: septate junction. Scale bars: 200 nm (**A**); 100 nm (**B–G**).

Scoring of the SJs’ architecture by semiquantitative criteria

In addition to the above measurements, we conducted an analysis of alterations in the architecture of SJs during development, using a scoring system of defined criteria. Five categories of SJ architecture were assigned accordingly: (i) single septa, (ii) short continuous array of septa (2–10 septa), (iii) discontinuous junctions containing short arrays, (iv) long continuous array of > 10 septa and (v) discontinuous junctions containing long arrays. Arrays of consecutive and regularly spaced septa were considered as continuous, while consecutive arrays of septa, separated by extended sections without visible septa, were considered as discontinuous. The following number of junctions were included in the semiquantitative evaluation: 13 junctions of mid-stage embryos (S13), 28 junctions of late-stage embryos (S16), 44 junctions of early marsupial mancae, 24 junctions of mid-stage marsupial mancae, 45 of late marsupial mancae, 55 and 33 junctions of postmarsupial mancae 3 days and 14 days after release from marsupium, respectively, and 20 junctions of adult animals.

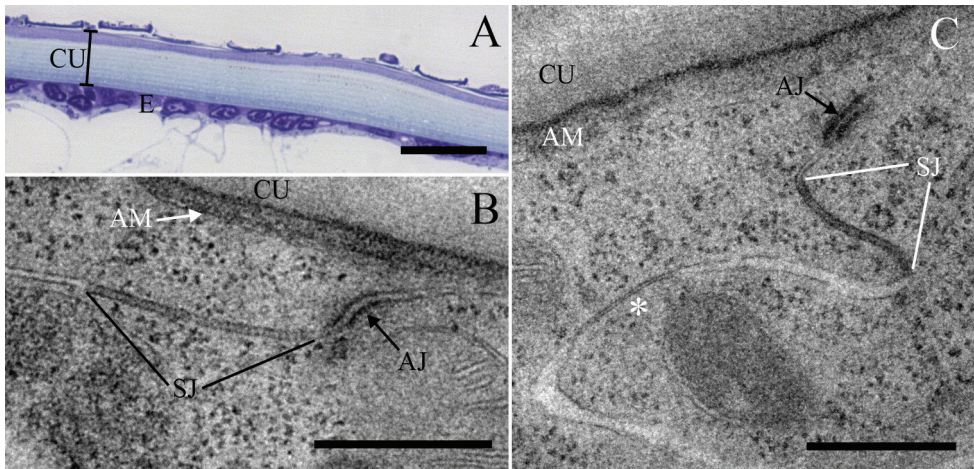


Figure 2. The ultrastructure of cell junctions in the epidermis of tergites of a *P. scaber* adult animal **A** semithin section of the tergite: The integument of adult animals consists of flattened epidermal cells covered by a thick cuticle **B** ultrastructure of an AJ and a pleated SJ with clearly resolved septa. SJs in adult animals are in the form of long continuous arrays of septa **C** an AJ in the subapical region of lateral cell membranes and a pleated SJ situated basally to the AJ. Further along the lateral membranes a close apposition of membranes is discernible (asterisk). Abbreviations: AJ: adherens junction; AM: apical membrane; CU: cuticle; E: epidermis; SJ: septate junction. Scale bars: 20 μm (**A**); 500 nm (**B, C**).

Results

The ultrastructure of intercellular junctions in the epidermis of tergites in intermoult adult animals

The epidermis of tergites in intermoult adult animals consists of flattened epithelial cells covered with a thick cuticle (Fig. 2A). Lateral membranes of epidermal cells are connected with AJs and basally adjacent to them, pleated SJs (Fig. 2B, C). Ultrastructurally, SJs are clearly revealed as “ladders” of consecutive electron dense septa that span the intercellular space and connect electron dense lateral membranes (Fig. 2B). As evident in some sections lateral membranes basally to SJs were closely apposed (Fig. 2C). SJs were generally arranged in long continuous arrays of septa that measure ~ 840 nm (Figs 2B, 3A). Sequential arrays of septa, separated by intercellular space without septa, were also evidenced and are termed discontinuous junctions thereafter. When discontinuous junctions were present, intermediate regions without septa were predominately short. Septa were arranged in a regular pattern, approximately 7 nm apart from each other (Fig. 3B), and ~ 5 nm thick (Fig. 3C). The width of intercellular space in the junction region was approximately 16 nm (Fig. 3D). AJs were located subapically, above SJs, and at a variable distance, ~ 140 nm from the apical membrane (Figs 2B, 4A). The length of AJs was ~ 120 nm (Fig. 4B). Two electron dense plaques

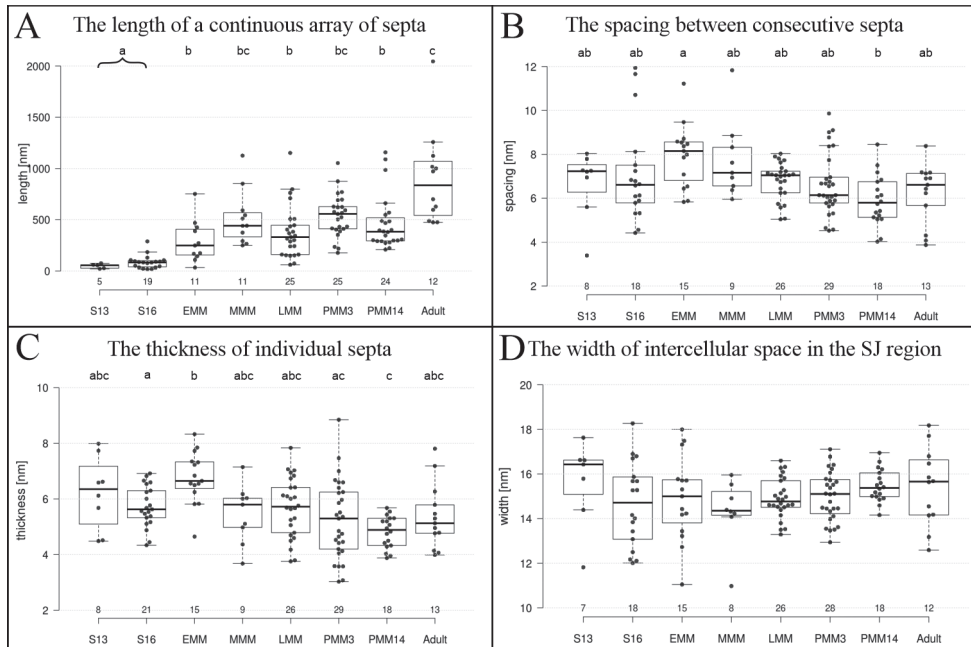


Figure 3. Measurements of the ultrastructural characteristics of SJs (A–D), graphically demonstrated by box-and-whiskers plots. Individual measurements are represented with dots and the numbers below the box-plots represent the number of measurements. The following stages were included in the analysis: mid-stage embryos S13 (S13), late-stage embryos S16 (S16), early-stage marsupial mancae (EMM), mid-stage marsupial mancae (MMM), late-stage marsupial mancae (LMM), postmarsupial mancae 3 days (PMM3), and 14 days (PMM14) after release from marsupium and adults. The letters above box-plots indicate significant differences between developmental stages (Mann-Whitney, $p < 0.05$). Measurements of the length of a continuous array of septa (A) in embryonic stages were pooled for statistical tests. Abbreviations: SJ: septate junction.

were seen on the cytoplasmic side of the adjacent cell membranes (Fig. 2B, C). The intercellular space in the AJ region was filled with material of intermediate electron density (Fig. 2B) and ~ 18 nm wide, which is significantly wider than the intercellular space of SJs (Fig. 4C).

Early stages of SJ formation and the structure of AJs in the embryonic epidermis

In this study, SJs with ultrastructurally discernible septa were first evidenced in the epidermis of mid-stage embryos (Fig. 5). While septa were not yet observed in mid-stage embryos of stage S10 (Fig. 5C) and S12, they were evidenced in S13 embryos (Fig. 5D) and in subsequent embryonic stages, including mid-stage embryos S14 (Fig. 5E) and late-stage embryos S16 (Fig. 5H) and S18 (Fig. 5I). Throughout these stages, we recorded single septa, continuous short arrays (Fig. 5D) and discontinuous junctions comprising short arrays of septa (Fig. 5E, H, I). The size of the longest continuous

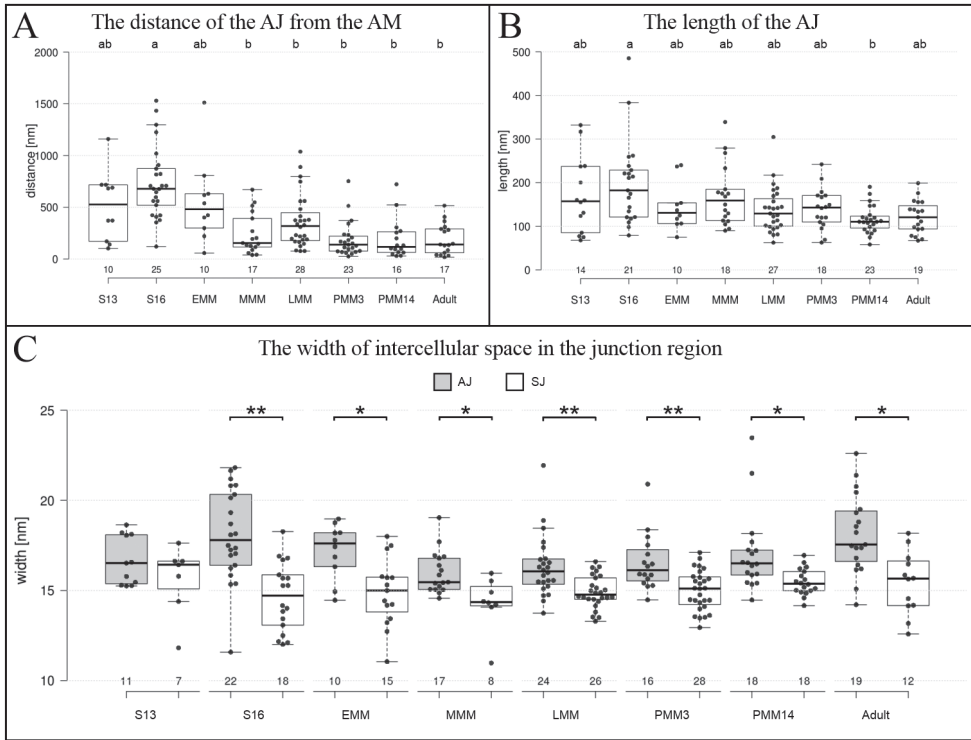


Figure 4. Measurements of the ultrastructural characteristics of AJs (**A**, **B**) and a comparison of the width of intercellular space in the AJs' and SJs' region (**C**), graphically demonstrated by box-and-whiskers plots. Individual measurements are represented with dots and the numbers below the box-plots represent the number of measurements. The following stages were included in the analysis: mid-stage embryos S13 (S13), late-stage embryos S16 (S16), early-stage marsupial mancae (EMM), mid-stage marsupial mancae (MMM), late-stage marsupial mancae (LMM), postmarsupial mancae 3 days (PMM3) and 14 days (PMM14) after release from marsupium and adults **A**, **B** the letters above box-plots indicate significant differences (Mann-Whitney, $p < 0.05$) between developmental stages **C** two sample Mann-Whitney tests were performed to determine statistically significant differences in the width of the AJs' and SJs' intercellular spaces of each developmental stage: $p < 0.01$ (*), $p < 0.001$ (**). Abbreviations: AJ: adherens junction; AM: apical membrane; SJ: septate junction.

septa arrays was ~ 60 nm in mid-stage embryos S13 and ~ 80 nm in late-stage embryos S16, but this difference is not statistically significant (Fig. 3A). In mid-stage and late-stage embryos, the spacing between consecutive septa was ~ 7 nm and septa thickness ~ 6 nm (Fig. 3B, C). In mid-stage embryos S13 the median width of intercellular space in the SJs' region was 16 nm and in the AJs' region 17 nm, while in late-stage embryos S16 it was 15 nm in the SJs' region and 18 nm in the AJs' region. The comparison of the width of intercellular space in both junctional regions showed a significant difference for S16 (Fig. 4C). AJs were ubiquitous in the epidermal epithelia of all analysed embryonic stages of *P. scaber* and ultrastructurally similar to those in other analysed

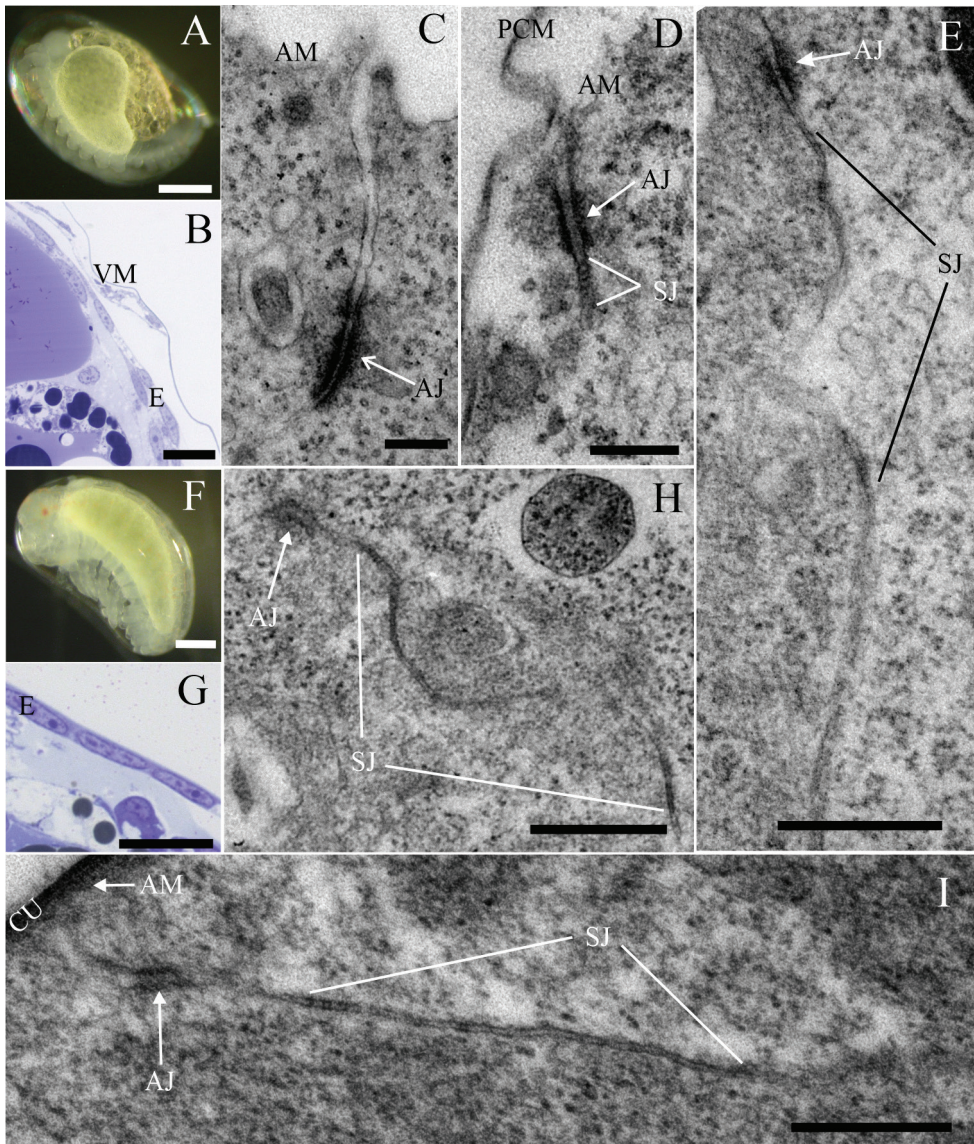


Figure 5. The ultrastructure of the dorsal body surface epidermal cell junctions in embryonic stages of *P. scaber* **A–E** samples of mid-stage embryos **A** the external morphology of mid-stage embryo S13 **B** semithin section of an S13 embryo: Epidermal cells on the dorsal part of the body are flattened **C** in mid-stage embryos S10, an AJ is evidenced between neighbouring epidermal cells. Septa of SJs are not observed **D** mid-stage embryos S13: the AJ is located subapically and its ultrastructure is similar to that in adult AJs. A short array of septa is evident just beneath the AJ **E** mid-stage embryos S14: A discontinuous SJ containing short arrays of septa **F–I** samples of late-stage embryos **F** late-stage embryo S16 **G** semithin section of S16 embryo: Epidermal cells of dorsal body surface are flattened **H, I** in late-stage embryos S16 (**H**) and S18 (**I**) SJs are mainly discontinuous and consist of short arrays of septa. Abbreviations: AJ: adherens junction; AM: apical membrane; CU: cuticle; E: epidermis; PCM: precuticular matrix; SJ: septate junction; VM: vitelline membrane. Scale bars: 0,2 mm (**A, F**); 20 μ m (**B, G**); 200 nm (**C, D**); 500 nm (**E, H, I**).

developmental stages and adult animals (Fig. 5C–E, I). AJs in embryonic stages were generally located more basally than in mancae and adults, ~ 530 nm from the apical cell surface in S13 and ~ 680 nm in S16 embryos (Figs 4A, 5C–E). The distance of AJs from the apical cell surface in embryos S16 is significantly larger in comparison to developmental stages from mid-stage marsupial mancae onwards (Fig. 4A). The length of AJs was ~ 160 nm in mid-stage embryos S13, and ~ 180 nm in late-stage embryos S16, which is larger than in all other stages and adults, but a significant difference is shown clearly between embryos S16 and postmarsupial mancae 14 days after release from marsupium (Fig. 4B).

Formation of long arrays of septa and remodelling of SJs is characteristic for early postembryonic developmental stages

We analysed the ultrastructure of AJs and SJs in the epidermis of marsupial manca stages (Fig. 6) to evaluate the differentiation of junctions after hatching from the vitelline membrane, i.e. at the beginning of the postembryonic development (early marsupial mancae) and at the stage of cuticle renewal (late marsupial mancae). Early-stage marsupial mancae, immediately after hatching from the vitelline membrane (Fig. 6A–D), was the only marsupial manca stage in which single septa were noted, however short continuous and discontinuous arrays of septa prevailed (Fig. 6C, D). In mid-stage marsupial mancae epidermis (Fig. 6E–H), the most common were long continuous arrays of septa (Fig. 6G, H), while short continuous and discontinuous arrays were less frequent. Interestingly, in late-stage marsupial mancae (Fig. 6I–L) discontinuous junctions, mainly those containing short arrays, were most often observed (Fig. 6K, L). A change in cell shape was also indicated in this stage, as cells were more cuboidal in contrast to flattened cells observed in previous and subsequent developmental stages (Fig. 6J). Data on the length of a continuous array of septa show a statistically significant increase in marsupial manca stages in comparison to embryos (Fig. 3A). The length of continuous arrays of septa was ~ 250 nm in early-, ~ 440 nm in mid- and ~ 330 nm in late-stage marsupial mancae (Fig. 3A). The spacing between consecutive septa was ~ 8 nm in early and ~ 7 nm in late and mid-stage marsupial mancae (Fig. 3B). Septa thickness was ~ 7 nm in early- and ~ 6 nm in mid- and late-stage marsupial mancae (Fig. 3C). The width of the intercellular space at the SJs' site was ~ 15 nm in early and late marsupial manca stages and ~ 14 nm in mid-stage marsupial mancae (Fig. 3D). The intercellular space within the AJs was ~ 18 nm in early-stage, ~ 15 nm in mid-stage and ~ 16 nm in late-stage marsupial mancae, in all stages significantly wider than the intercellular space within SJs (Fig. 4C). The location of AJs was generally subapical, ~ 480 nm from the apical membrane in early-, ~ 150 nm in mid- and ~ 320 nm in late-stage marsupial mancae (Figs 4A, 6C, G, K, L). In comparison to late embryos S16, the AJs of mid- and late-stage marsupial mancae are located closer to the apical cell surface (Fig. 4A). The size of AJs was ~ 130 nm in early, ~ 160 nm in mid and ~ 130 nm in late marsupial manca stage (Fig. 4B).

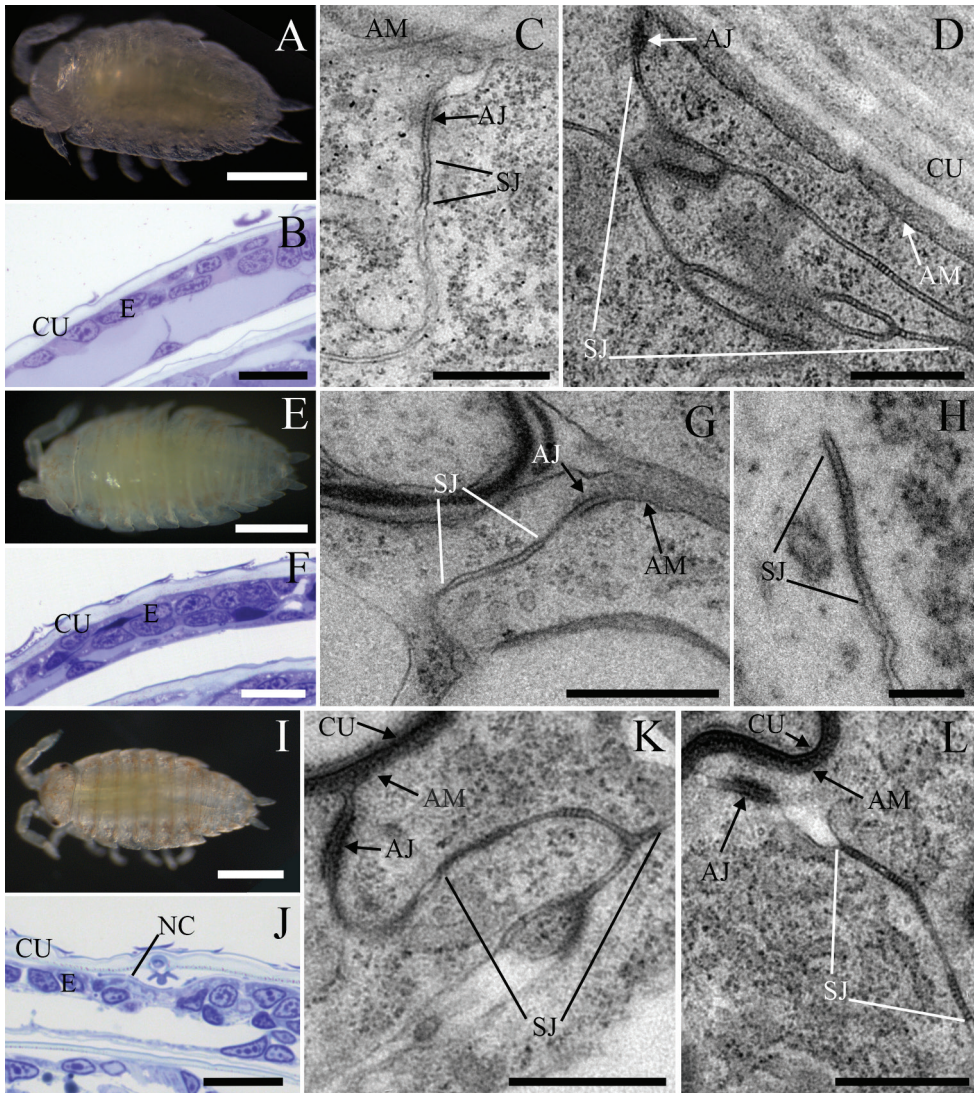


Figure 6. The ultrastructure of cell junctions between epidermal cells of tergites in marsupial manca stages of *P. scaber* **A–D** samples of early marsupial manca **A** early marsupial manca **B** semithin section of manca: epidermal cells of the tergite are flattened and covered by a cuticle **C** a short continuous array of septa is evident beneath the AJ **D** discontinuous junctions containing short arrays of septa are often evidenced along lateral cell membranes of neighbouring cells **E–H** samples of mid-stage marsupial manca **E** mid-stage marsupial manca **F** semithin section of manca: The epidermis of the tergite consists of flattened cells, which are covered by a cuticle **G** the junctional complex consists of a subapically located AJ and basally adjacent to it a long continuous SJ **H** continuous long array of septa **I–L** samples of late marsupial manca **I** late marsupial manca **J** semithin section of late manca epidermis: Epidermal cells are not as flat as in all other analysed developmental stages. Detachment of the cuticle and formation of a new cuticle reveal the renewal of the exoskeleton **K** discontinuous junctions containing short arrays of septa are often evidenced beneath the AJ in late marsupial manca stage **L** discontinuous long arrays of septa are rarely observed in late-stage marsupial manca. Abbreviations: AJ: adherens junction; AM: apical membrane; CU: cuticle; E: epidermis; NC: new cuticle; SJ: septate junction. Scale bars: 500 µm (**A**, **E**, **I**); 20 µm (**B**, **F**, **J**); 500 nm, (**C**, **D**, **G**, **K**, **L**); 200 nm (**H**).

Continuous and discontinuous long arrays of septa are characteristic for SJs in postmarsupial manca stages

We analysed samples of postmarsupial mancae to evaluate later stages in the differentiation of SJs and the effect of the change of environment, from the marsupium to the external environment (Fig. 7). Postmarsupial mancae 3 and 14 days after release from marsupium were analysed. SJs were predominately evidenced as long continuous arrays or discontinuous junctions containing long arrays and an increase in abundance of septa in comparison to previous developmental stages was observed (Figs 3A, 7C, E, F). The length of continuous arrays of septa was ~ 560 nm in mancae 3 days after release from marsupium and ~ 380 nm in mancae 14 days after release from the marsupium

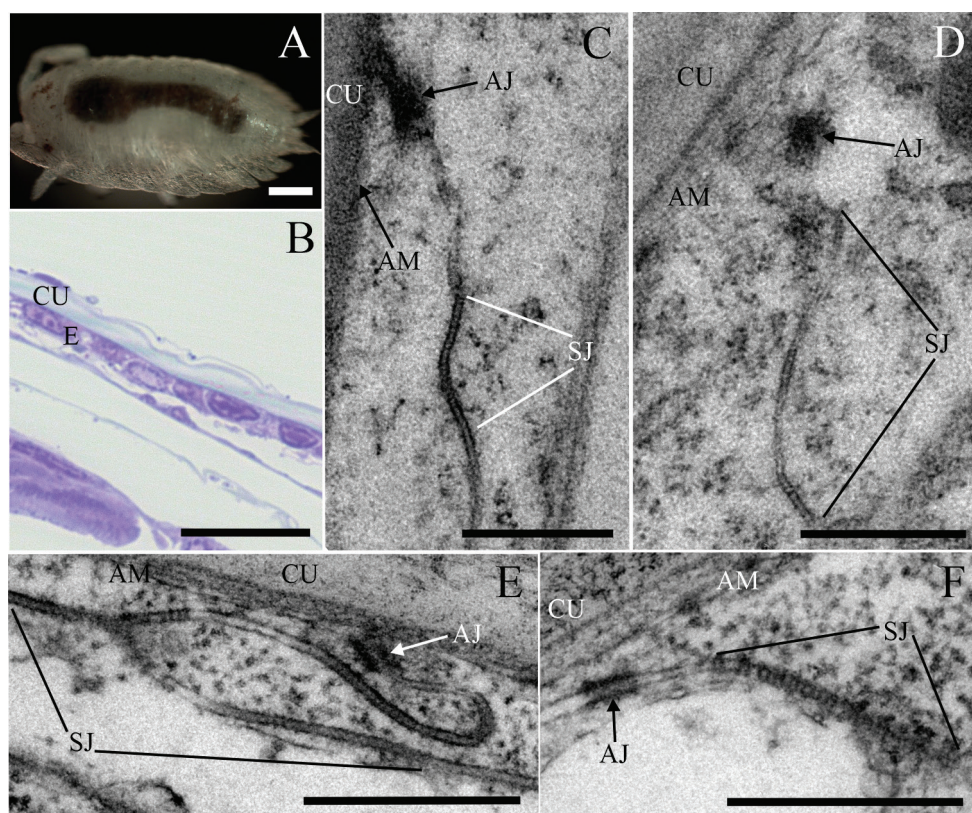


Figure 7. The ultrastructure of cell junctions in the epidermis of tergites in *P. scaber* postmarsupial mancae **A** external morphology of postmarsupial manca **B** semithin section of epidermis: Flattened epidermal cells are covered by a cuticle that is not yet as thick as in adult animals **C–D** AJs and SJs of postmarsupial mancae 3 days after release from marsupium **C** a long continuous array of septa is evident between neighbouring cells **D** discontinuous junctions containing short arrays of septa are rarely observed **E–F** epidermal cell junctions of postmarsupial mancae 14 days after release from marsupium **E** discontinuous junction containing long arrays encompasses the lateral membranes **F** long continuous SJs are often observed. Abbreviations: AJ: adherens junction; AM: apical membrane; C: cuticle; E: epidermis; SJ: septate junction. Scale bars: 200 μ m (**A**); 20 μ m (**B**); 500 nm (**C–F**).

(Fig. 3A). Septa were ~ 6 nm apart (Fig. 3B) and ~ 5 nm thick (Fig. 3C) in both postmarsupial manca stages. The width of the intercellular space in the SJs' region was approximately 15 nm, and significantly wider in the AJs' region, ~ 16 –17 nm (Fig. 4C). The AJs were ~ 140 nm and ~ 120 nm from the apical membrane in postmarsupial mancae 3 days and 14 days after release from marsupium, respectively (Fig. 4A). The approximate length of AJs was 140 nm in postmarsupial mancae 3 days after release from marsupium and 110 nm in postmarsupial mancae 14 days after release from marsupium (Figs 4B, 7E, F).

A summary of the alterations in the architecture of SJs in the epidermis of *P. scaber* throughout development is presented according to our semiquantitative analysis (Fig. 8). SJs are first established in mid-stage embryos S13 and are evidenced as single septa and short continuous or discontinuous arrays of septa. The established SJ architecture is maintained through embryonic stages. In early postembryonic development SJs gradually expand, until they are generally configured as long continuous arrays of septa in mid-stage marsupial mancae. The stage of late marsupial manca reintroduces the predominance of discontinuous junctions containing short arrays of septa. This remodelling of SJs coincides with exoskeletal cuticle renewal in late marsupial mancae. SJs in postmarsupial manca stages exhibit predominately continuous long arrays of septa or discontinuous junctions containing long arrays of septa. In the epidermis of intermoult adults SJs are generally comprised of long continuous arrays of septa or discontinuous junctions containing long arrays of septa, while single septa and short arrays of septa were not detected.

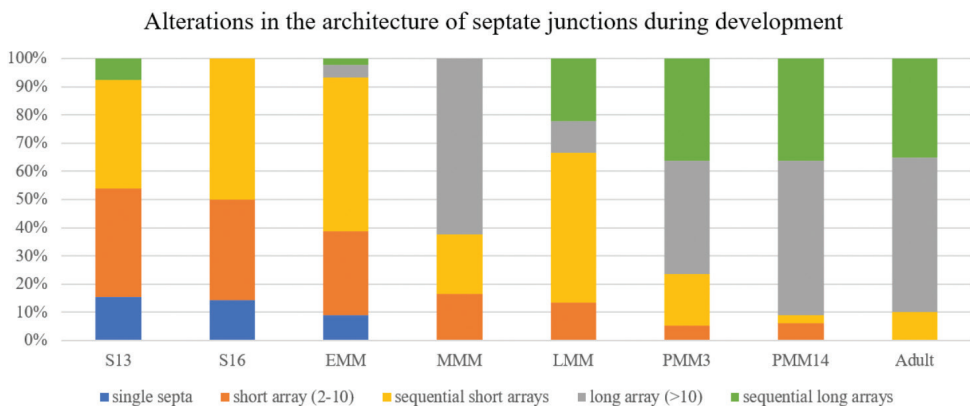


Figure 8. Semiquantitative analysis of the alterations in the architecture of SJs during development. The analysis includes mid-stage embryos S13 (S13), late-stage embryos S16 (S16), early-stage marsupial mancae (EMM), mid-stage marsupial mancae (MMM), late-stage marsupial mancae (LMM), postmarsupial mancae three days after release from the marsupium (PMM3), postmarsupial mancae 14 days after release from marsupium (PMM14), and adult animals.

Discussion

The ultrastructure of SJs in *P. scaber* tergite epidermis

The ultrastructure of SJs in tergite epidermis of intermoult adult *P. scaber* was characterised and compared with SJs in the hindgut epithelium of the same species (Bogataj et al. 2018) and with pleated SJs in different ectodermal epithelia of adult specimens of other arthropod species (Happ and Happ 1970; Lane and Skaer 1980; Noirot-Timothee and Noirot 1980).

The epidermal and hindgut epithelia of *P. scaber* are both monolayered, ectodermal in origin and covered by a chitinous cuticle, but they have several morphological and ultrastructural specialisations reflecting their different functions. Epidermal cells are flattened and covered by a thick and mineralised cuticle, which forms a protective barrier (Hild et al. 2008; Seidl and Ziegler 2012). On the other hand, hindgut epithelial cells are isodiametric and covered by a cuticle that is not mineralised. The hindgut cuticle is ten times thinner than the exoskeletal cuticle and displays a less pronounced organisation of lamellae, a pattern appearing due to helicoidal arrangement of chitin-protein fibres (Bogataj et al. 2018). Lateral cell membranes in the epidermis are slightly convoluted, however they are intensely interdigitated in the hindgut epithelium. In addition, in the hindgut the apical and basal membrane form membrane labyrinths, which are associated with numerous mitochondria in the papillate region (Bogataj et al. 2018). These ultrastructural features of the hindgut epithelium are in accordance with the transporting and osmoregulatory role of the two functional regions of the hindgut, the anterior chamber and papillate region, respectively (Hryniewiecka-Szyfter and Storch 1986; Hames and Hopkin 1989; Bogataj et al. 2018). Comparative ultrastructure of pleated SJs in the epidermis and hindgut epithelium of *P. scaber* shows considerable differences in the architecture of SJs. In comparison to the mainly long continuous SJs in the epidermis, SJs in the hindgut epithelium are even more extensive, mainly discontinuous and intensely convoluted due to the interdigitations of lateral plasma membranes. Such interdigitations are more pronounced in the papillate region of the hindgut (Bogataj et al. 2018). In the hindgut, several dilations of the intercellular space were evident regularly in between the septal arrays (Bogataj et al. 2018). In the epidermis, the dilations of the intercellular space are not as numerous, however they can span over larger areas below the SJ. In the epidermis, the intercellular space of characteristic width for SJs was filled in some cases with finely granulated material of medium electron density and septa were not clearly resolved. Another distinction in the ultrastructure is related to microtubules in the vicinity of SJs. Bogataj et al. (2018) reported an abundance of microtubules in the vicinity of SJs in both regions of the hindgut, but we did not observe this in the epidermis. The role of the hindgut epithelium in transport and osmoregulation suggests a pronounced need for the restriction of paracellular transport (Hryniewiecka-Szyfter and Storch

1986; Hames and Hopkin 1989) and the differences in the architecture of SJs in the epidermis and in the hindgut epithelia, are likely related to the functional differences between both ectodermal epithelia.

Pleated SJs in the tergite epidermis of adult intermoult *P. scaber* characterised in this study consist of long continuous or discontinuous arrays of electron dense septa. Discontinuous pleated SJs have already been described in arthropods by Noirot-Timotheé and Noirot (1980). Ziegler (1997) studied the ultrastructure of the sternal epithelium in *P. scaber* and reported also on the apical cell contacts between cells. Cell junctions of sternal epithelial cells comprise AJs, SJs and additional cell contacts, characterised by a reduced distance between plasma membranes. Subsequent research by Ziegler and Merz (1999) has confirmed these contacts as gap junctions. We report a similar architecture of apical cell junctions in the *P. scaber* tergite epidermis, and we observed the locations of apposition of plasma membranes basally to SJs, which are similar to gap junctions described in Ziegler (1997).

A general width of 15 nm of the intercellular space in the region of SJs, as described here for the epidermis of *P. scaber*, has previously been reported for pleated SJs in arthropods (Lane and Skaer 1980; Noirot-Timotheé and Noirot 1980). Regularly spaced septa, ~ 6 nm apart, are representative for SJs in the epidermis of *P. scaber*. While this regularity has also been reported in the epidermis of different arthropods (Lane and Skaer 1980; Noirot-Timotheé and Noirot 1980), a study by Lane and Skaer (1980) has shown that septa in different tissues of the locust *Schistocerca gregaria* are either regularly or irregularly spaced. A spacing between septa of pleated SJs has been reported to be in the range of 16–20 nm in different arthropod epithelia (Happ and Happ 1970; Lane and Skaer 1980; Noirot-Timotheé and Noirot 1980). Noirot-Timotheé and Noirot (1980) defined that the reported spacing of septa in their study is attributed to the center-to-center spacing, however other authors did not specify the method of measurements. In our study, the septal distance was specified as the space between the edges of consecutive septa. Distinct methods of measurements could contribute to the difference of septal distance between our results and data from other studies. Our results in *P. scaber* tergite epidermis have revealed an average septal thickness of 5 nm, however for other arthropods, a septal thickness of 2–9 nm has been reported (Lane and Skaer 1980; Noirot-Timotheé and Noirot 1980). These results indicate that septal thickness and spacing in pleated SJs varies considerably in different arthropod epithelia, while the width of intercellular space in SJs' region is rather invariable.

In addition to SJs, epidermal cells of *P. scaber* are circumferentially surrounded and connected by subapically located AJs. Our analysis of AJs in the tergite epidermis of adult specimens of intermoult *P. scaber* has shown a ubiquitous presence as well as a uniform ultrastructural appearance of two regularly spaced electron dense plaques on the cytoplasmic side of lateral plasma membranes. Similar results have previously been reported by Bogataj et al. (2018) in epithelia of both hindgut regions of the same species. In our analysis, we have also shown a variability in the length of individual AJs and in the location with regard to the apical membrane.

The formation of SJs in the epidermis of *P. scaber* during embryonic and postembryonic development involves a gradual increase in the abundance of the septa and the formation of continuous arrays

A detailed study of ultrastructural differentiation of pleated SJs in arthropod epithelia has been performed in the common fruit fly, *Drosophila melanogaster* by Tepass and Hartenstein (1994). The molecular composition of SJs in the fruit fly has been characterised in several studies (Izumi and Furuse 2014; Hall and Ward 2016; Rice et al. 2021). The biogenesis of SJs in the hindgut epithelium of *P. scaber* was characterised by Bogataj et al. 2019. Only fragmentary data on the formation of SJs, referring mainly to specific developmental stages are available for other arthropod species (Locke 1965; Hagopian 1970; Caveney and Podgorski 1975; Noirot-Timothee and Noirot 1980; Lane and Swales 1982). We show in this paper that the ultrastructural differentiation of SJs in the epidermis of *P. scaber* during embryonic and postembryonic development is characterised by consecutive steps of junction assembly, beginning with the formation of a few electron-dense septa near the AJs in mid-embryonic stage S13. Differentiation of SJs during further development includes gradual elongation of septa arrays and formation of continuous arrays of septa. A gradual increase of the extent of SJs during late embryonic and postembryonic development was evidenced also in the hindgut epithelium of *P. scaber* (Bogataj et al. 2019). A similar sequence of events in SJs' biogenesis has been reported in different epithelia of *D. melanogaster*, including the epidermis, trachea, pharynx, oesophagus, inner layer of proventriculus, hindgut and salivary glands (Tepass and Hartenstein 1994; Tepass et al. 2001). Taken together, these results suggest a common principle of SJs' biogenesis in *D. melanogaster* and *P. scaber* ectodermal epithelia, but there are considerable differences in the timelines of SJs' formation in these two species. In *D. melanogaster*, SJs' biogenesis occurs in the range of several hours, while in *P. scaber* the duration of these events is measured in days. The core SJ proteins are expressed early in *D. melanogaster* embryonic development and are localised along the lateral membrane by stage 12, after roughly 9 hours of embryogenesis (Hall and Ward 2016; Rice et al. 2021). In fruit fly embryos that have reached stage 14, the first septa have been identified (Tepass and Hartenstein 1994; Rice et al. 2021). Gradually, more septa are formed, and studies of functionality have shown that the paracellular barrier is established in stage 15, after 12 hours of embryonic development (Paul et al. 2003; Rice et al. 2021). In the next 10–12 hours regularly arranged septa are formed and are described as a hallmark of stage 17 embryos, the final stage of *D. melanogaster* embryonic development (Rice et al. 2021). Mature SJs are attributed to the first instar larva of *D. melanogaster* (Tepass and Hartenstein 1994), but further postembryonic development was not followed in this respect (Tepass and Hartenstein 1994; Rice et al. 2021). In *P. scaber* epidermis, initial septa are formed by approximately the 17th day of embryogenesis and additional septa are added throughout embryonic development. When the embryo hatches from the vitelline membrane (on ~ 25th day of embryogenesis) and the epidermis is thereafter exposed to the marsupial fluid, the arrays of septa become more abundant. During postembryonic development

of marsupial mancae, SJs are further elongated due to additional septa incorporation and long arrays of septa are as frequent as short arrays. Approximately on the 35th day of development, mancae are released from the marsupium to the external environment and the architecture of SJs resembles that in adult animals, but the septa are still not as abundant as in adults.

Our results show that the intercellular space in the region of SJs was not significantly different in analysed developmental stages. As for the thickness and spacing of consecutive septa, our analysis did not reveal a clear pattern of changes corresponding to developmental stages. The main difference between immature and mature SJs is in the abundance and distribution of septa. To the best of our knowledge there are no other reports on measurements of SJs' structural characteristics during SJs' biogenesis in relation to tissue morphogenesis. There are however some studies which offer fragmentary data on SJs in specific developmental stages of various arthropodal species (Locke 1965; Hagopian 1970; Caveney and Podgorski 1975).

The remodelling of SJs is characteristic for *P. scaber* late marsupial mancae epidermis (shown in this study) and for the hindgut epithelium (Bogataj et al. 2019). SJs' remodelling in the hindgut epithelium is more conspicuous and is characterised by a considerable shortening of the junction, while in the epidermis continuous junctions are transformed into discontinuous and shorter arrays of septa. The main physiological process in late marsupial manca stage that could be associated with SJs' remodelling in analysed ectodermal epithelia of *P. scaber* is cuticle formation. However, for the hindgut, Bogataj et al. (2019) have also suggested a possible contribution of the feeding pause in moulting mancae to SJs' remodelling. In general, data on the remodelling of SJs on the ultrastructural level are sparse. Reversible remodelling of pleated SJs was examined by Khan and Saleuddin (1981) in the renal epithelium of adult gastropod *Hellisoma* (Mollusca: Gastropoda), and the changes were attributed to experimental exposure of animals to a hypoosmotic medium. In the tracheal epithelium of *D. melanogaster*, a link between the formation of the chitin matrix and SJs has been established, identifying specifically the role of SJ proteins in the secretion of chitin deacetylases Serp and Verm (Wang et al. 2006; Luschnig et al. 2006; Nelson et al. 2010). Deacetylases Serp and Verm are required to form and modify a chitin cylinder that serves as a template for accurate tracheal morphogenesis, but their absence in *serp* and *verm* mutants has no effect on the barrier function of tracheal epithelia (Luschnig et al. 2006). In addition, Serp and Verm as well as SJ components are required for the correct formation and the rigidity of the epidermal cuticle. The absence of Serp and Verm affects the shape of the body. The study by Lamb et al. (1998) also showed that correct SJs' formation in *D. melanogaster* is essential for the structure of embryonic cuticle. The absence of Coracle, a structural protein of SJs, affects the localisation of other SJs' components and the ultrastructure of SJs. A consequence of Coracle absence is that the embryonic cuticle is thinner and the epicuticle fails to adhere to the procuticle, resulting in two detached layers (Lamb et al. 1998). It is known that epidermal cell shape changes during moulting cycle in crustaceans (Compère et al. 2004; Dillaman et al. 2013),

and it was also indicated in this study that the height of the epidermis is enlarged in late marsupial mancae. It seems likely that SJs' remodelling in late marsupial mancae accompanies cell shape changes.

Our measurements indicate a decrease in the distance of AJs from the apical membrane at the transition to postembryonic development while results on the length did not show significant changes in relation to development. For *D. melanogaster*, Tepass and Hartenstein (1994) showed that AJs in ectodermal epithelia are fully formed shortly after gastrulation and that their ultrastructure does not significantly change throughout the development. Tepass and Hartenstein (1994) reported a generally 20–60 nm long band of AJs in the larval epidermis of *D. melanogaster*. The constant ultrastructure of the AJs is consistent with their role in the establishment of cell polarity, formation of apical and basolateral plasma membrane domains, and maintenance of tissue integrity (Tepass et al. 2001; Payre 2004).

Conclusions

Pleated SJs in the tergite epidermis of adult intermoult *P. scaber* characterised in this study consist of long continuous or discontinuous arrays of electron dense septa and are less extensive than in the hindgut epithelium of the same species. We consider that distinct ultrastructures of SJs reflect different functions of both epithelia and suggest also differences in the paracellular barriers.

We determined the first stage of septa formation in the epidermis of mid-stage embryo S13, where single septa and short arrays of septa were detected. Further formation of SJs in the epidermis of *P. scaber* during embryonic and postembryonic development involves a gradual increase in the abundance of the septa and the formation of continuous arrays. The enlargement of SJs in the epidermis is most pronounced at the transition from embryonic to postembryonic development and after the release of mancae from the marsupium. A similar sequence of SJs' biogenesis has also been reported in the hindgut of the same species. The subsequent addition of septa until long arrays of septa are formed appears to be representative of SJs' biogenesis in the ectodermal epithelia of arthropods.

The late marsupial manca stage represents a period of SJs' remodelling and conversion of continuous junctions to discontinuous and shorter arrays. Similar, but more pronounced remodelling of SJs was described in the hindgut epithelia of *P. scaber* in the same developmental stage. We consider that these changes in SJs' architecture in the analysed ectodermal epithelia of *P. scaber*, are related to the processes of moulting.

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Intersexuality in a natural population of the terrestrial isopod *Porcellio scaber*

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Abstract

Intersex phenotypes are rarely observed in natural isopod populations and their expression is typically associated with infection of *Wolbachia*, a reproductive parasite that manipulates arthropod reproduction. During an intensive sampling effort of a natural population of the isopod *Porcellio scaber*, an adult individual was isolated that expressed both male and female traits. The intersex individual exhibited clearly developed external male genitalia and carried multiple eggs in its brood pouch. No *Wolbachia* infection could be identified in this individual, a result that needs to be approached with caution due to suboptimal DNA preservation for diagnostic PCR assays. *Wolbachia* were, however, detected in two adult females of the same population, and appear closely related to isolates that infect other terrestrial isopod species. This is the first demonstration that intersex phenotypes can arise under natural conditions in *P. scaber*.

Keywords

Intersex phenotypes, Isopoda, sex-determination, *Wolbachia*

Introduction

Sex-determination mechanisms regulate the sexual differentiation of organisms and are highly diverse across the animal kingdom. Sex-specific differentiation can rely on external environmental cues but can also be solely regulated by the segregation of genetic factors (Bull 1985; Zarkower 2001). In a wide range of arthropod species, sex-determination is controlled by multiple genetic factors (Cordaux et al. 2011; Moore and Roberts 2013). For instance, populations of the house fly *Musca domestica* carry several male-determining chromosomes and an additional female sex-determination locus (Dübendorfer et al. 2002; Hediger et al. 2010). Intersex individuals that express both male and female traits spontaneously arise at low frequencies in natural populations of certain arthropod species (Narita et al. 2010). In isopods, the appearance of intersex individuals is often linked to infection of *Wolbachia*, probably one of the most widespread invertebrate-associated bacteria (Bouchon et al. 1998, 2009; Engelstädter and Hurst 2009; Cordaux et al. 2011, 2012). *Wolbachia* are maternally transmitted endosymbiotic bacteria that infect the reproductive tissues of arthropods and nematodes (Werren 1997). *Wolbachia* spread within host populations by manipulating host reproduction in multiple ways (Engelstädter and Hurst 2009). In isopods, *Wolbachia* can feminize genetic males into functional phenotypic females (Rigaud et al. 1991; Bouchon et al. 1998; Cordaux et al. 2004). However, under certain conditions, *Wolbachia*-mediated feminization can be incomplete and intersex individuals can arise (O'Neill et al. 1997; Herran et al. 2020).

To date, *Wolbachia*-infected individuals have been described in at least 39 isopod species, with all *Wolbachia* isolates belonging to the *Wolbachia* B- or F-supergroup (Bouchon et al. 1998; Cordaux et al. 2001; Zimmermann et al. 2021). *Wolbachia*-mediated feminization of the common pill woodlouse *Armadillidium vulgare* is well understood and has become a model system to study the evolution of sex-determination pathways (Rigaud et al. 1997; Herran et al. 2020). Here, the androgenic hormone synthesized by the androgenic gland is responsible for the formation of male gonads. It has been experimentally shown that the implantation of an androgenic gland into females can induce functional sex reversal depending on the timing of implantation (Suzuki and Yamasaki 1997). These findings indicate that all *A. vulgare* individuals possess the necessary genetic basis that is required for male and female sexual differentiation (Rigaud et al. 1997).

In genetic males, *Wolbachia* likely inhibit the development of the androgenic gland by either targeting the androgenic hormone promotor or the androgenic hormone receptor hereby feminizing the individual (Rigaud et al. 1997). Incomplete feminization has been associated with low densities of *Wolbachia* during embryonal development (O'Neill et al. 1997; Bouchon et al. 2009; Cordaux et al. 2011; Herran et al. 2020). Low *Wolbachia* densities can be the result of increased temperature levels as has been shown for *A. vulgare* (Herran et al. 2020). Despite the widespread occurrence of *Wolbachia* in natural isopod populations (Bouchon et al. 1998, 2009), intersex individuals have been observed in only a limited number of isopod species,

including *A. vulgare*, *Armadillidium album*, *Porcellio laevis*, *Sphaeroma rugicauda* and *Ligia oceanica* (Juchault et al. 1991; Bouchon et al. 1998; Ben Nasr et al. 2010).

Here, we present the first record of an intersex *Porcellio scaber* collected from a natural population in Snellegem (Belgium) in August 2020. The individual carried a large number of eggs in its brood pouch, and can thus be considered as a functional female. However, the individual also possessed clearly developed external male genitalia.

Materials and methods

Isopod collection

We used cuboid pitfalls (25 cm × 7.5 cm × 8 cm) containing an approximately 1 cm bottom layer of plaster to collect isopods in Brugge, Snellegem, and Vleteren (Parmentier et al. 2021). The plaster was moisturized to prevent the desiccation of all trapped isopods, so that we could collect the isopods alive; all isopods were collected one week after installation of the pitfalls and preserved on 70% ethanol. Individuals were preserved on 70% ethanol for three months before phenotyping.

Diagnostic PCR detection of *Wolbachia* infection

In addition to the intersex individual, we also isolated four adult females that exhibited normal sexual differentiation from the Snellegem population. Sterility was maintained by working in a biological safety cabinet. After washing the specimens twice in sterile water for 1 min, DNA was extracted from whole bodies using the Quick-DNA Universal kit (BaseClear, the Netherlands). DNA integrity was tested by amplifying a fragment of cytochrome c oxidase subunit I (*COI*) using the LCO1490 and HC02198 primers (Folmer et al. 1994). A standardized PCR approach was performed to test *Wolbachia* infection in the five *P. scaber* samples (one intersex individual and four normal females) using DreamTaq DNA Polymerase (Life Technologies Europe BV). The standard primers of the multilocus sequence typing system for *Wolbachia* were used to amplify fragments of the *wsp*, *ftsZ*, *hcpA*, *coxA*, and *gatB* genes, standard molecular markers to detect *Wolbachia* infection (Baldo et al. 2006). *Wolbachia*-infected *Myrmica scabrinodis* workers were used as positive controls. The *hcpA* gene fragment was Sanger sequenced for one of the *Wolbachia*-infected *P. scaber* females that exhibited normal sexual differentiation (MACROGEN Europe B.V.).

Results

From a collection of 7,814 individuals, we found a *P. scaber* individual in the Snellegem population that carried eggs and, although egg viability was not ascertained, could be considered as a functional female (Fig. 1). However, in contrast to female isopods



Figure 1. Ventral view of the intersex specimen of *P. scaber* with multiple eggs in the marsupium (1) and a male pleon (2).

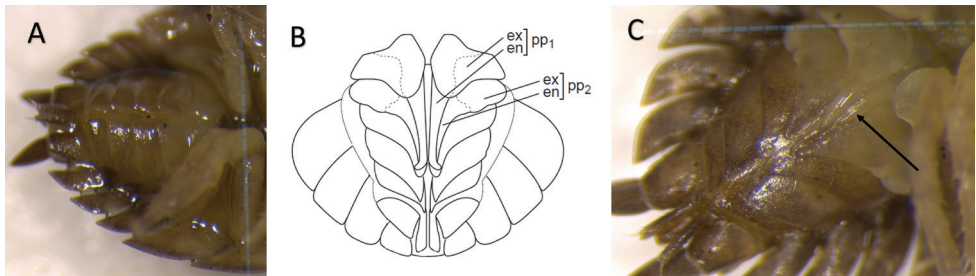


Figure 2. **A** ventral view of the pleon of a normal female *P. scaber* **B** drawing of the ventral view of the male pleon of *A. vulgare*, similar in morphology to *P. scaber* **C** ventral view of the pleon of intersex *P. scaber* with the endopodite of the first pleopod marked with an arrow. Abbreviations: **pp1** first pleopod; **pp2** second pleopod; **ex** exopodite; **en** endopodite (**B** drawn from Shultz 2018).

(Fig. 2A), this individual possessed an elongated and well-developed endopodite of the first pleopod, a canonical male sexual characteristic in isopods (Fig. 2C). In addition, the exopodites and endopodites of the first and second pleopods were also shaped differently than females, forming a male pleon (Fig. 2B, C). In the marsupium of the individual multiple eggs were found, comparable in number to normal females. We did not manage to amplify a fragment of the *COI* gene for the intersex individual, while PCR amplification was successful for four normal Snellegem females that were collected at the same time. This apparent discrepancy in DNA integrity might be due to a longer exposure to air for the intersex individual during photography. Using

diagnostic PCR assays, we were unable to identify a *Wolbachia* infection of the intersex individual. *Wolbachia* infection was confirmed in two of the four normal females. Sanger sequencing of the *hcpA* gene fragment revealed a 100% identity to a *Wolbachia* isolate that was previously retrieved from the isopod *Helleria brevicornis* (Sicard et al. 2014). The partial *hcpA* gene sequence was deposited in the GenBank database under the accession number OM459769.

Discussion

Intersex individuals are rarely observed in natural populations of arthropods (Narita et al. 2010). The intersex phenotype of the current study appears to be present at an extremely low frequency in natural populations of *P. scaber*. However, additional intersex individuals were possibly overlooked, because they were not carrying visible eggs at the time of collection. One study previously described a similar, but not identical, intersex phenotype in *P. scaber* where three specimens displayed greatly reduced male genitalia and were expected to be functional females according to the authors (Sassaman and Garthwaite 1984). In contrast to our individual, these individuals came from laboratory stocks and not from a wild natural population. In addition, our individual did not show reduced genitalia.

It is tempting to speculate that incomplete *Wolbachia*-mediated feminization caused the intersex phenotype in this individual. *Wolbachia* are widespread in *P. scaber*, infecting populations across Europe (Bouchon et al. 1998). We uncovered that the natural Snellegem population was also infected. Previous work has shown that both males and females of *P. scaber* carry *Wolbachia* (Bouchon et al. 1998). Moreover, interspecific transfer of feminizing *Wolbachia* into *P. scaber* revealed that its sex-determination mechanisms can be manipulated by the reproductive parasite and can result in intersex individuals under controlled laboratory conditions (Bouchon et al. 1998). However, it remains uncertain whether the *Wolbachia* variants that naturally infect *P. scaber* are able to feminize genetic males. Unfortunately, we could not bring more clarity to this outstanding question due to our inability to confirm *Wolbachia* infection in our intersex individual. Due to the suboptimal preservation of the individual (Marquina et al. 2021), DNA degradation was likely too severe and interfered with our diagnostic PCR assays, a hypothesis that is supported by our inability to amplify a *COI* gene fragment. Currently, we cannot exclude that other mechanisms, such as pollution- or virus-induced developmental abnormalities, caused the formation of a functional female *P. scaber* with male genitalia (Juchault et al. 1991; Ford 2012).

Acknowledgements

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Bug cemetery: a case study of terrestrial isopod distribution on a brick wall in the Czech Republic

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Abstract

Although terrestrial isopods (Oniscidea) are primarily soil- and surface-dwelling invertebrates, they can also be found on tree trunks and walls. This study evaluated distribution patterns of terrestrial isopods on a brick wall during the first hours of night in autumn. Four species of terrestrial isopods were recorded with *Armadillidium versicolor* being the dominant one. Terrestrial isopods were distributed from ground level up to a height of 2 m, but preferred a 70–80 cm height band. The highest number of active individuals was observed 3 h after astronomical dusk. Potential predators of terrestrial isopods were abundant during the same time and at the same height.

Keywords

Isopoda, Oniscidea, synanthropic habitat, vertical distribution, woodlice

Introduction

Terrestrial isopods (Crustacea, Isopoda, Oniscidea) inhabit soil generally and feed on dead and rotting organic matter. They can be found in upper soil layers including the litter layer (Cole 1946). They shelter under bark, logs and stones or they are true troglobionts. There are several species climbing on vegetation as reported from tropical areas, such as Central America (e.g., Van Name 1936), Central (Schmidt 1999) and South Africa (Glazier and Kleynhans 2015) or St. Helena Island (Dutton and Pryce

2018). In Europe, there are only a few anecdotal reports about the presence of terrestrial isopods on trees, walls, and inside tree hollows for species of the genus *Armadillidium* (e.g., *A. pictum* Brandt, 1833, *A. depressum* Brandt, 1833, *A. pulchellum* (Zenker, 1798), *A. vulgare* (Latreille, 1804)), the genus *Porcellio* (*P. scaber* Latreille, 1804, *P. spinicornis* Say, 1818), *Oniscus asellus* Linnaeus, 1758, and occasionally other species (Růžicka et al. 1991; Alexander 2008, 2011; Gregory 2009; Božanić et al. 2013; Tracz 2013; Boeraeve et al. 2021). This above-ground activity is sometimes related to heavy rains (Abbott 1918; Standen 1921; Cole 1946) or spring floods (Fig. 1). However, a recent systematic inventory of arthropods in oak canopies in Norway revealed five species of terrestrial isopods with high prevalence of *A. pictum* (Thunes et al. 2021). Sutton (1972) noted the presence of *P. scaber* on tree trunks and brick walls feeding on green algae.

Although the nocturnal presence of *P. scaber* on trees is commonly known (Sutton 1972; Cloudsley-Thompson 1977; Warburg 1993), there are only a few systematic studies. The first one was by Brereton (1957), who systematically searched for terrestrial isopods on tree trunks near Oxford for two years; he reported four species found on tree trunk bases but only two of them (*P. scaber* and *O. asellus*) also at eye-level height (~ 1.8 m). During the year, isopods were the most numerous on trees in spring and in September/October. Brereton (1957) confirmed that *P. scaber* overwinters in moss pillows at the base of the trunk and the species climbs on branches during late summer: he found them in high numbers at 5.5 m height in early September. High abundance of terrestrial isopods in moss on the base of tree trunks were caused by downward migration of isopods of the canopy and the trunk, as confirmed with the use of trunk traps (Brereton 1957). The surprising opposite pattern of seasonal migration on trees for overwintering of *P. scaber* was suggested by Fritsche (1934; Vandel 1962). Prevailing descent migration from trees during a year was revealed for *Spherillo* spp. and *Trichoniscus* spp. in New Zealand (Moeed and Meads 1983), indicating breeding in canopies.

Another exhaustive study of *P. scaber* living on trees near Den Haag, the Netherlands, was published by Den Boer (1961), who studied its activity in the same years as Brereton (1957), 1953–1954 and 1953–1955, respectively. He described parameters of daytime shelters on the bark for *P. scaber*. He found that very few isopods, active during the night on tree trunks with such shelters, travel down to hide in the litter layer during daytime. Another interesting finding (recorded using the capture-mark-recapture method) is that although isopods are present on the bark during the whole night, activity of each individual isopod spans only approximately one hour (Den Boer 1961).

Sixty years later, aspects of distribution of terrestrial isopods on tree trunks were presented at the 11th International Symposium on Terrestrial Isopod Biology by Mechthold et al. (2021). They highlighted phenological differences between tree trunk active populations of *P. scaber* and *O. asellus* during the year.

Beside these studies on the activity of terrestrial isopods on tree trunks, there is only one paper studying distribution of isopods on walls. Cloudsley-Thompson (1958) counted terrestrial isopods on a stone wall at night and found that their numbers decrease with increasing wind speed. His explanation was that wind inhibits their activity due to a reduction of air humidity.



Figure 1. Springtails and terrestrial isopods climbing on tree trunks to avoid drowning during a spring flood. *Trachelipus rathkii* (Brandt, 1833), indicated by pink arrow, *Protracheoniscus politus* (C. L. Koch, 1841), indicated by orange arrow, and *Porcellium conspersum* (C. L. Koch, 1841), indicated by red arrow, are visible. Litovelské Pomoraví PLA (Czech Republic), 30 March 2006 (photographs IHT).

We also received anecdotal observations of terrestrial isopods climbing on walls of buildings during the night. In this study we present data on the distribution of terrestrial isopods on a brick wall in the Czech Republic to study (1) whether there is a temporal pattern in the distribution of particular species of terrestrial isopods, (2) whether its distribution depends on the air temperature or humidity, and (3) whether the distribution of predators corresponds with the distribution of terrestrial isopods.

Materials and methods

After a short pilot survey, we selected a study wall on which we found a high number of active terrestrial isopods during the night. This particular brick wall was found on the outskirts of the town of Kostelec na Hané (Czech Republic) at the local cemetery (49°31'06.0"N, 17°03'44.6"E). The length of the wall is 190 m and its height is ~ 2.5 m. The first 4 m of the wall is plastered, the rest are bare bricks standing on a 50 cm high stone foundation (Fig. 2A). Terrestrial isopods and their potential predators were studied along a 30 m long transect of the non-plastered part at the outward side of the wall (exposed to the east, i.e., influenced by the prevailing south-eastern wind) from the base to a height of 2 m. The wall was bordered by a freshly ploughed agricultural field and generally surrounded by an urban and agricultural landscape. The nearest forest is located at ~ 2 km. The nearest street lamp was located ~ 20 m away, but the studied side of the wall was not illuminated by artificial light.



Figure 2. **A** the studied brick cemetery wall **B** *Armadillidium versicolor* climbing on the wall (photographs NW).

Following Brereton (1957), the month of October (2019) was chosen as the best time to study terrestrial isopod activity. Previous observations indicated the highest abundance of terrestrial isopods between 21:00–23:00 h (i.e., 1.5–3.5 h after astronomical dusk which was at 19:38 on the first observation day; Fig. 2B). Therefore, terrestrial isopods and other invertebrates were observed daily from 20 to 26 October. Each daily observation started at $\sim 21:15 (\pm 15 \text{ min})$ and lasted 90 min. At the start of each observation, we measured air humidity, air temperature, and light intensity (lux). For each observed individual, we noted species identity, time of observation, and height above the ground with a Dictaphone. Numbers of predatory invertebrates, i.e., centipedes, spiders, and harvestmen, were also noted to evaluate the pattern of distribution of potential predators. Observations were made using a hand torch with white light, since we were not interested in the behaviour of the animals, but only their position. No fleeing reaction was noted during the research. No individuals were collected during the observations. A few individuals for identification were taken a few days before the start of the first observation.

All data was entered into MS Excel, and we used CANOCO 5 (Šmilauer and Lepš 2014) for statistical analysis. Numbers of individuals of the different terrestrial isopod species were used as species variables. As environmental variables we used height (cm), air humidity (%), air temperature ($^{\circ}\text{C}$), time after astronomical dusk (minutes), and numbers of individuals of predators (summed numbers of centipedes (Chilopoda), spiders (Araneae), and harvestmen (Opiliones)). We used a redundancy analysis (RDA) for visualizing the relationship between number of terrestrial isopods

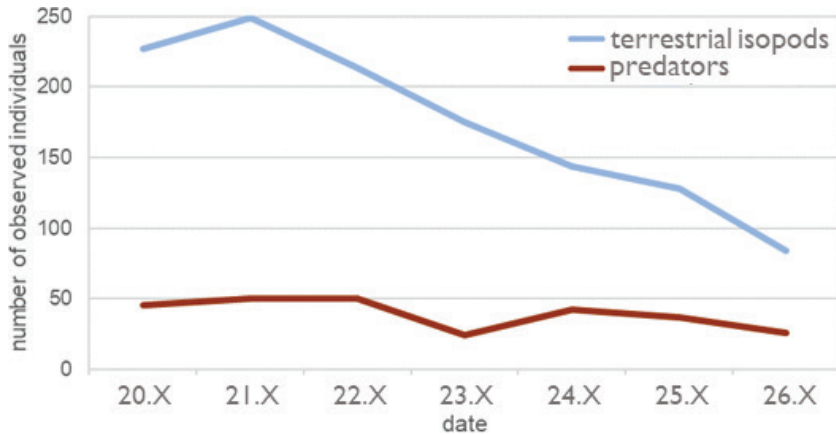


Figure 3. Number of invertebrates recorded per day on the wall during a 90-min observation during October 2019.

per species and environmental variables. We did not incorporate the effect of light intensity in the analysis, because measured values were very low and varied between 0 and 2 lx. Environmental variables that significantly explained variation in the terrestrial isopod distribution were used to calculate predictive GAM models (normal distribution of data).

Results

In total, 1221 terrestrial isopods belonging to four species were observed. By far the most numerous was *Armadillidium versicolor* Stein, 1859 (1020 individuals), followed by *Porcellio spinicornis* (112 ind.), *Armadillidium vulgare* (85 ind.), and *Porcellionides pruinosus* (Brandt, 1833) (4 ind.). Altogether, 266 spiders and only two centipedes and nine harvestmen were observed. The number of observed terrestrial isopods and predators decreased during the sampling period (Fig. 3), which could be due to the decreasing recorded temperature. However, this correlation was not significant (Pearson's $r = 0.37$ and $p = 0.414$ for temperature and number of observed terrestrial isopods, Pearson's $r = 0.48$ and $p = 0.276$ for temperature and number of observed predators). The highest mean numbers of individuals for (almost) all species were recorded at a height of 70–80 cm (Fig. 4).

The RDA for predicting the distribution of terrestrial isopods using environmental variables (Fig. 5) was statistically significant (pseudo- $F = 28.3$, $p = 0.002$). The first and the second axes explained 47.2% and 3.6%, respectively of the variability in terrestrial isopod distribution. The strongest predicting variables were number of predators (explaining 35.2%, pseudo- $F = 74.8$, $p = 0.002$) and the height above the ground (explaining 12.2%, pseudo- $F = 31.8$, $p = 0.002$), whereas time after astronomical dusk

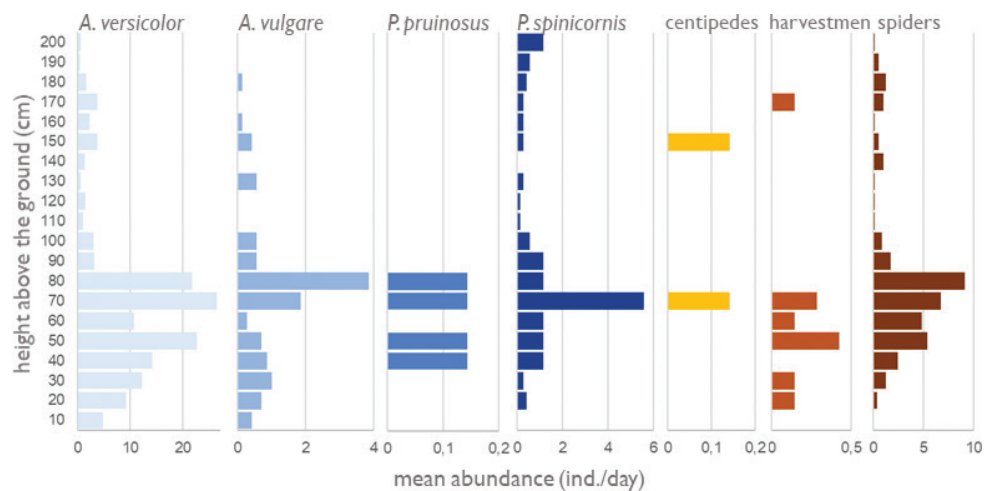


Figure 4. Vertical distribution of terrestrial isopod species and predators on the wall per day in 90-min observation.

and air temperature explained less than 2% of the variability in the terrestrial isopod distribution (1.8%, pseudo-F = 4.8, $p = 0.002$ and 1.9%, pseudo-F = 5.3, $p = 0.01$, respectively). The effect of air humidity was not significant (0.2%, pseudo-F = 0.8, $p = 0.456$). The presence of predators is a good predictor for the presence of terrestrial isopod species (Table 1, Fig. 6A), although *P. spinicornis* was most numerous at sites with medium numbers of predators.

Based on GAM models, the number of terrestrial isopods was significantly predicted by the height of the record on the wall for all species except *P. pruinosis* (Table 1), the three other species were most numerous at a height of 70–80 cm above the ground (Fig. 6B). The time after astronomical dusk was a significant predictor for the number of observed individuals of *A. versicolor* (Table 1), which reached the highest numbers ~ 3 h after dusk (Fig. 6C). The air temperature had no significant effect on the prediction of the numbers of observed individuals of single terrestrial isopod species (Table 1).

Table 1. Summary of fitted Generalised Additive Models for environmental variables predicting the numbers of observed terrestrial isopods on the wall during a 90-min observation in October 2019. Significant effects in bold. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

Predictors	predators (ind.)			height (cm)			time after sunset (min)			air temperature (°C)		
Response	R2[%]	F	p	R2[%]	F	p	R2[%]	F	p	R2[%]	F	p
<i>A. versicolor</i>	54.3	71.2	***	19.4	14.4	***	6.4	4.1	*	4.3	2.7	n.s.
<i>A. vulgare</i>	29.2	24.8	***	10.1	6.7	**	4.5	2.9	n.s.	4.3	2.7	n.s.
<i>P. pruinosis</i>	29.3	24.8	***	3.4	2.1	n.s.	0.8	0.5	n.s.	3.1	1.9	n.s.
<i>P. spinicornis</i>	23.4	18.3	***	10.3	6.9	**	2.4	1.5	n.s.	0.7	0.4	n.s.

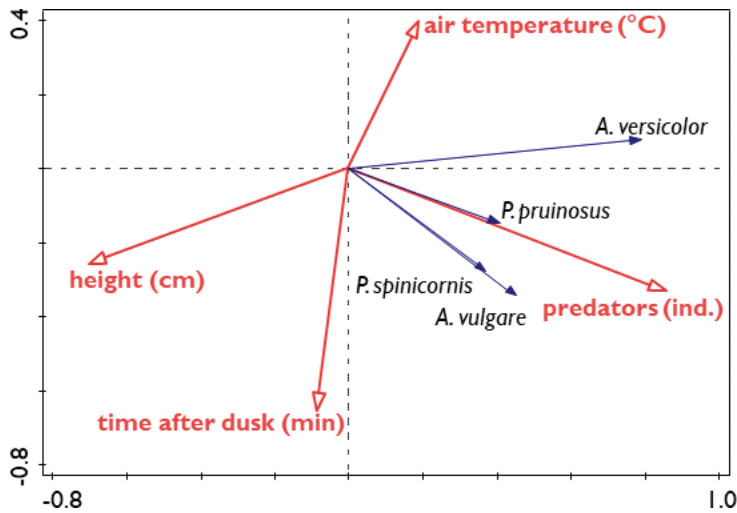


Figure 5. RDA-biplot for the distribution of different species of terrestrial isopods (blue arrows) on the wall during a 90-min observation in October 2019 and environmental variables (red arrows). Only environmental variables with a significant effect on terrestrial isopod distribution are presented.

Discussion

We present data on the distribution of four species of terrestrial isopods on a wall at night in autumn 2019 in the Czech Republic. The distribution of the most numerous species was significantly explained by the height on the wall and for one species there was also a significant correlation between the number of observed individuals and the time after sunset. Distribution of all species had a significant relationship with the presence of predators, which mainly consisted of spiders.

All species of terrestrial isopods found on the studied wall are common inhabitants of cities in the Czech Republic (Orsavová and Tuf 2018). The absence of *P. scaber* is surprising because it is frequently found on walls of buildings in the nearby city of Olomouc (pers. obs.) and it was reported from walls several times (e.g., Den Boer 1961), including brick walls (Meinertz 1944). The only species found in this study and reported before from (limestone) walls is *P. spinicornis* (Sutton 1972; Boeraeve et al. 2021).

The preferred height of all species was ~ 70–80 cm, with low numbers of animals at 60 cm (Fig. 4). It is necessary to say that at a height of 60 cm there was a small prominent lintel between the lower stones and the upper brick parts of the wall (see Fig. 2A). For some reason, animals (both terrestrial isopods and spiders) were more numerous above and below this brick lintel. Higher numbers of observed individuals could be related to higher humidity near the lintel (catching rainfall) or small accumulation of debris including excrements (see Fig. 2B); probably without the presence of the lintel, distribution of terrestrial isopods on the wall would be less unequal.

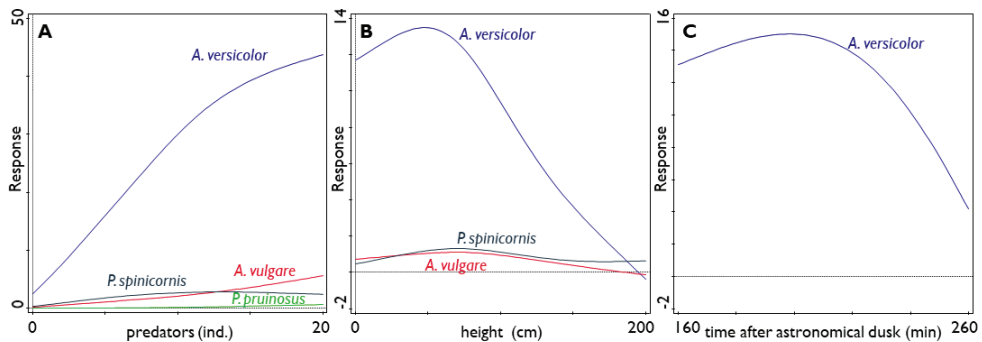


Figure 6. Significant response (see Table 1) of terrestrial isopod numbers per species to **A** number of predators **B** height on the wall **C** time after dusk during a 90 min interval in October 2019.

The observed temporal pattern, with the highest number of observed individuals at approximately 21:30 h, is in accordance with the temporal activity pattern observed for *P. scaber* in the Oxford study (Brereton 1957). This species was active there from 19:00 till 03:00 h, with the highest observed activity around 23 h, indicating that its activity increased continuously the first hours after sunset. Brereton (1957) observed individuals of *P. scaber* mainly on well-structured bark of oaks and sycamores. On the other hand, Den Boer (1961) reported the highest numbers of *P. scaber* after sunset and before dawn. He supposed that individuals climb the tree trunks actively each evening from litter around the tree base because they are not able to find shelter on the smooth bark of studied aspens. The wall we studied is old with partly eroded ground between the bricks with many crevices offering a lot of shelter. We do not suppose that terrestrial isopods climb on the wall from shelters at or near the soil every night and therefore our data support the view of Brereton (1957) that the terrestrial isopods find daytime shelter on the wall.

We found a weak effect of air temperature and no effect of air humidity on the activity of terrestrial isopods. Cloudsley-Thompson (1958), as well as Den Boer (1961), reported an effect of air humidity in explaining the vertical activity of terrestrial isopods. However, we did not find this effect, which may be an artefact of the low variability in air humidity during our research (58–70%, one night of 46%), but it is also necessary to say that the genus *Armadillidium* is less vulnerable to low humidity levels than, e.g., *P. scaber* (Cloudsley-Thompson 1977) and has a higher drought resistance (Dias et al. 2013). On the other hand, Brereton (1957) did not find effects of air humidity, nor air temperature, on the activity of *P. scaber*.

The strongest predictive power for the numbers of observed terrestrial isopods on the wall was the number of observed predators. Centipedes, spiders, and harvestmen are known isopod predators (Cloudsley-Thompson 1958; Sutton 1970; Santos and Gnaspini 2002). The observed predatory individuals were the centipede *Lithobius forficatus* (Linnaeus, 1758), the harvestman *Phalangium opilio* Linnaeus, 1761, and the spiders *Nuctenea umbratica* (Clerck, 1757) and *Steatoda grossa* (C. L. Koch, 1838). Of course, terrestrial isopods do not prefer places with high predatory pressure, but

the reason for the presence of predators at specific heights with high numbers of terrestrial isopods is probably due to accessibility of their prey or overall suitability of environmental conditions there. Nevertheless, we can conclude that high abundance of predators at a particular height could be a good predictor for terrestrial isopods on walls.

It is not yet clear exactly why terrestrial isopods are found on walls, but the search for algae as food source is most plausible. Brereton (1957) experimentally confirmed consumption of *Pleurococcus* algae from the tree bark by *P. scaber*. However, in the same experiment, algae were not consumed by *O. asellus* (Brereton 1957). We therefore encourage research that investigates feeding habits of terrestrial isopods on walls and on trees!

In conclusion, we observed four species of terrestrial isopods present on the wall during the several hours after sunset. They were distributed along the complete studied height (up to 2 m), but they preferred a height of ~ 0.75 m above the soil surface. Similar spatiotemporal patterns were recorded for spiders as their potential predators. We suppose that the terrestrial isopods shelter in fissures and crevices between bricks and that they are resident on the wall. It will be very useful to study their gut contents to discover what food is consumed, which will probably explain their distribution.

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Daily and seasonal time partitioning in surface activity of *Porcellio albinus* (Isopoda, Oniscidea) in the arid region of Zarat (Gabes, Tunisia)

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Abstract

The terrestrial isopod *Porcellio albinus* is a burrowing species, dwelling in the desert of south Tunisia. Field studies were carried out in the coastal area of Zarat, Tunisia, to examine the surface activity rhythm of *P. albinus* in relation to daily and seasonal variations in environmental conditions. The activity of *P. albinus* was followed once a month, from November 2012 through October 2013. Hourly capture frequency was compared across the different seasons of the year. *Porcellio albinus* is a strictly nocturnal species showing a nycthemeral rhythm regulated by the rhythmic and natural variations of the duration of the dark period. A positive correlation is observed between the circadian rhythm of the locomotor activity of the species and the duration of the dark period outside its burrow, *P. albinus* has a single daily activity peak. Individuals concentrated their activity in the first part of the night in winter and in the second part in summer. This peak is more spread out in spring and autumn. The differences in the activity rhythm of *P. albinus* between different seasons may be determined by two important factors, namely temperature and the length of the dark period.

Keywords

Burrowing species, circadian rhythm, desert, epigeic activity, woodlice

Introduction

Activity patterns in terrestrial organisms are crucial for migration, survival, and reproduction (Engenheiro et al. 2005). Hence, it is an important and relevant ecological parameter. Terrestrial isopods are an important component of the soil macrofauna and are restricted in their activity in space and time to conditions where air and substrate moisture are relatively high. Therefore, terrestrial isopods are mainly nocturnal and show strong seasonal patterns in activity, especially in arid regions. At night they appear from their hiding places and are able to wander in dry areas where they are not usually found during the day. In general, this is true for the majority of terrestrial isopods, but there are exceptions such as *Armadillidium vulgare* Latreille, 1804 and *Hemilepistus reaumurii* (Milne-Edwards, 1840), which are more resistant to desiccation compared to other species (Edney 1951; Ayari et al. 2016), and can appear out of their burrows in full sunlight (Cloudsley-Thompson 1951; Ayari et al. 2018). Extensive and detailed studies on these activity patterns in different species of terrestrial isopods unravel species-specific activity patterns deviating from strict nocturnal activity. For example, the semi-terrestrial burrowing isopod *Tylos spinulosus* Dana, 1853 shows activity along the beach depending on the tide (Jaramillo et al. 2003); *Armadillidium vulgare* is active in the morning hours in open habitats (Cloudsley-Thompson 1951) and the forest species *Porcellium conspersum* (C. Koch, 1841) is active mainly during the day in spring (Tuf and Jeřábková 2008). *Hemilepistus reaumurii* occurs in desert regions and is active mostly during the hours of daylight with a bimodal pattern during warm months and a unimodal surface activity pattern during cold months (Ayari et al. 2018). The daily epigeic activity of terrestrial isopods depend mainly on the light-dark regime and to a lesser extent on air humidity and temperature (Tuf and Jeřábková 2008).

Living often alongside *H. reaumurii* (Medini-Bouaziz et al. 2017a), the burrowing desert isopod *Porcellio albinus* Budde-Lund, 1885 is one of the few terrestrial isopod species well adapted to the sandy desert habitats of North-Africa (Monod 1932; Medini-Bouaziz 2002). Despite its wide distribution, there have been few studies on this species' ecology. Only Linsenmair (1989, 2007) provided some information on its reproduction and behaviour, while the density and the distribution of *P. albinus* burrows were studied by Fraj et al. (2008) in the Kebili region (Tunisia). Recently, Medini-Bouaziz (2018) and Medini-Bouaziz et al. (2017a, b) carried out field studies on the abiotic factors determining the spatial distribution of burrows, the population dynamics, and the reproductive aspects of *P. albinus* in the Zarat area in Tunisia. Previous studies on behavioural and reproductive strategies of *Porcellio* species (Medini-Bouaziz et al. 2017b; Medini-Bouaziz 2018) in the Zarat region showed that specimens of *P. albinus* were observed outside their burrows at night and never during the day in contrast to *H. reaumurii*. However, these patterns have never been studied in detail. Therefore, we aim to investigate the nocturnal rhythm of the species and the environmental factors that influence it. We investigated the time partitioning of surface activity during night hours and its variation across the seasons to understand the surface activity rhythm of *P. albinus* under natural conditions and contribute to the knowledge of this widespread species' ecology.

Materials and methods

Study area

The study site is located in the sandy coastal area of Zarat, south of Gabès, Tunisia (33°40'N, 10°21'E (DDM)). The area is dominated by *nebkas*, morphological structures that were formed following an accumulation of sand brought by the wind and trapped by an obstacle (Medini-Bouaziz et al. 2017a). This area is situated at 1.5 km from the coastline. The climate is arid, i.e., rainfall is irregular and ranges between 100 and 200 mm per year (Genin et al. 2006), temperatures are usually high with considerable seasonal variation. The proximity of the study area to the sea moderates summer temperatures and therefore they do not exceed 28 °C on average in summer and 13 °C in winter (Genin et al. 2006).

Terrestrial isopod activity and environmental variables

Seasonal activity of *P. albinus* was investigated in the field in winter 2012, spring, summer, and autumn 2013. Recordings were performed at night in a rectangular area of 1,000 m² (100 m × 10 m) which was subdivided into ten corridors of 10 m × 1 m. Sampling was done one day every month for 24 hours. Sampling took place every two hours between and incorporating sunset and sunrise starting at 16 h in winter and spring and 17 h in summer and autumn. White flashlights were used to count and capture the individuals of *P. albinus*, while walking back and forth in every corridor. All individuals captured were used for a study on the reproductive cycle of *P. albinus* (see Medini-Bouaziz et al. 2017b). We are aware that this could lead to a small underestimation of the activity patterns at later time step during the night, but since activity periods of terrestrial isopods are relatively short, we do not expect that this could have a large impact on the results (Den Boer 1961). All ten corridors could be sampled in one hour. Only individuals whose size reached or exceeded 8 mm were counted and captured, those whose size is less than 8 mm (equivalent to less than 2 months old) do not leave their burrows (Medini-Bouaziz et al. 2017b).

We calculated the duration of surface activity as the period between the first and the last observed individual per night. This study was carried out simultaneously with the study from Medini-Bouaziz et al. (2017b) on population dynamics and reproduction of *P. albinus*.

At the same time, the most important environmental parameters (average temperature, wind speed, air humidity, dew point, and cloud cover) were registered every two hours, based on weather data for the Zarat region provided by the AccuWeather Az-Zarat (2013) at the day of sampling.

Porcellio albinus surface activity is assessed by its capture frequency which corresponds to the ratio between the number of individuals collected per time slot and the total number of individuals collected for all time slots.

Statistical analysis

To study the variation in surface activity between seasons, we used analysis of variance (ANOVA). We used Principal Component Analysis (PCA), with surface activity of *P. albinus* during the night as response variable to understand the importance of temporal (season and time) and environmental variables (temperature, wind speed, moisture, dew point and cloud cover) for *P. albinus* surface activity at night. All the statistical tests were applied at a confidence level (p-value) of 0.05 and performed using the XLSTAT 2018.6 software, used as a Microsoft Excel plug-in. A Pearson correlation test was used to investigate the strength of the linear relationship between length of the surface activity of *P. albinus* and the length of the dark period. A Friedman test is used to test the inter-seasonal difference observed in the duration of the surface activity period of *P. albinus*.

Results

Daily surface activity

The length of the surface activity of *P. albinus* was correlated with the length of the dark period ($r = 0.874$, $p < 0.05$) (Fig. 1). During the whole sampling period from November 2012 through October 2013, *P. albinus* showed strict nocturnal activity: individuals, whose size is equal to or exceeds 8 mm (Medini-Bouaziz et al. 2017b), began to emerge from their burrows after dusk (26 ± 4 min in March 2013 to 106 ± 6 min in September 2013) and returned to their shelters before dawn (17 ± 5 min in July 2013 to 110 ± 3 min in January 2013). The duration of the nocturnal surface activity of *P. albinus* varied from month to month. The shortest activity period and longest activity period were respectively in June and December coinciding with the shortest and longest dark period (Fig. 1).

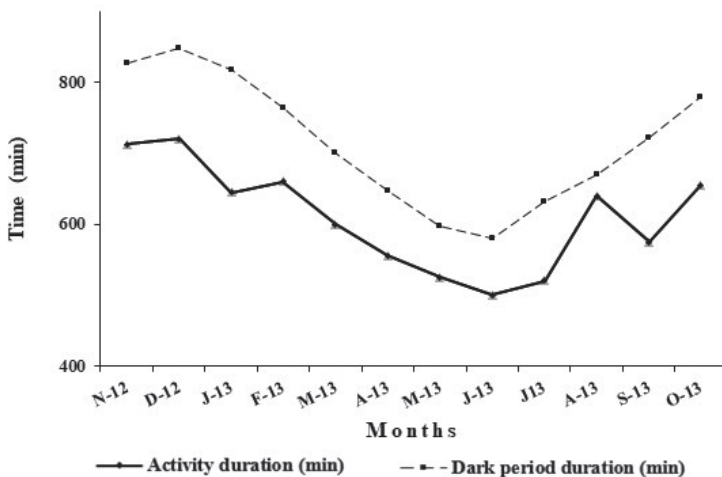


Figure 1. Daily surface activity variation of *Porcellio albinus*. Surface activity deduced from the mean time interval between the first and last observed isopod.

Seasonal surface activity

In total, 317 individuals of *P. albinus* were recorded and collected. The surface activity of this species shows a surface activity period that is longer in winter (675.0 ± 39.7 min) than in the other seasons, but this difference is statistically insignificant (Table 1). In winter, *P. albinus* concentrated its activity in the first part of the night, when the temperature was high enough, reaching its maximum at $\sim 21:00$ h (Fig. 2). In summer, *P. albinus* shifted its activity to the second part of the night, with a peak at $\sim 05:00$ h when temperature decreased. In spring and autumn, *P. albinus* surface activity was evenly distributed throughout the night. Table 2 shows the dependence of frequency on temperature, moisture, dew point, and wind speed. Capture frequency did not depend on Cloud cover or amount of rainfall.

Table 1. Coefficient of regression (R) below the diagonal and p values above the diagonal for pairwise comparison of *P. albinus* seasonal activity duration between the different seasons.

	Winter	Spring	Summer	Autumn
Winter	0	0.586	0.232	1.000
Spring	-1.333	0	0.921	0.586
Summer	-2.000	-0.667	0	0.232
Autumn	0.000	1.333	2.000	0

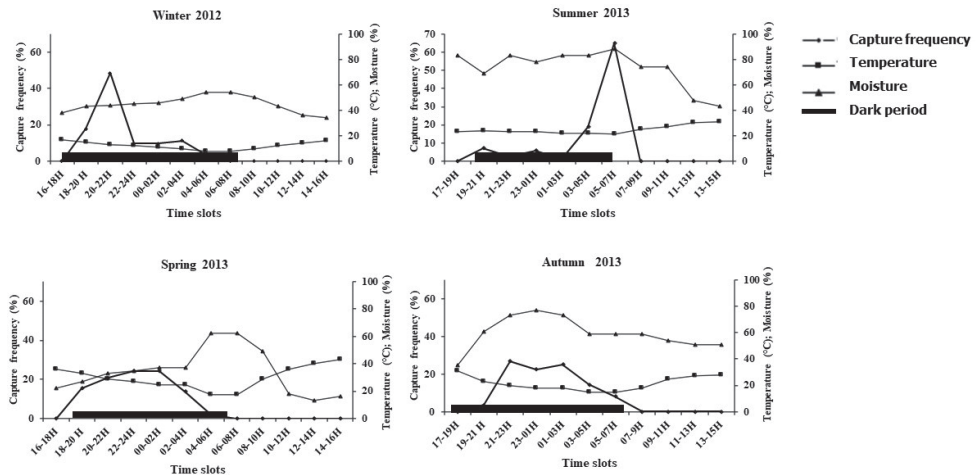


Figure 2. Temporal distribution of the surface activity of *P. albinus* during the four seasons in relation to the time of the day. Surface activity was deduced from the number of captured individuals. The continuous thick horizontal line indicates the dark period.

Table 2. Results of the Fiedman test investigating the dependency of capture frequency on different environmental variables.

	Temperature (°C)	Moisture (%)	Wind speed (Km/h)	Cloud cover (%)	Dew point (°C)	Rain (%)
R ²	0.5202	0.6330	0.3233	0.1884	0.9305	0.2000
F	10.1211	16.0993	4.4595	2.1666	125.0361	2.3333
p value	0.0001	< 0.0001	0.0111	0.1143	< 0.0001	0.0955

The Principal Component Analysis (PCA) gives more insight into the important environmental variables that influence capture frequency and thus surface activity of *P. albinus*. The first (F1) and second (F2) PCA axes explain 42.46% and 29.51% of the variance respectively with a cumulative percentage of 71.98 (Fig. 3A). Almost half of the variables are correlated to F1. Cloud cover, dew point, rain, and temperature as well as the effect of the summer season are projected positively along F1. Along F2, wind speed, the first trapping hour (H1) and the effect of the spring season are positively correlated; on the other side, capture frequency, the effect of the autumn season, and moisture are negatively correlated along F2. The effect of the winter season and temperature strongly correlates to F3 (Fig. 3B)

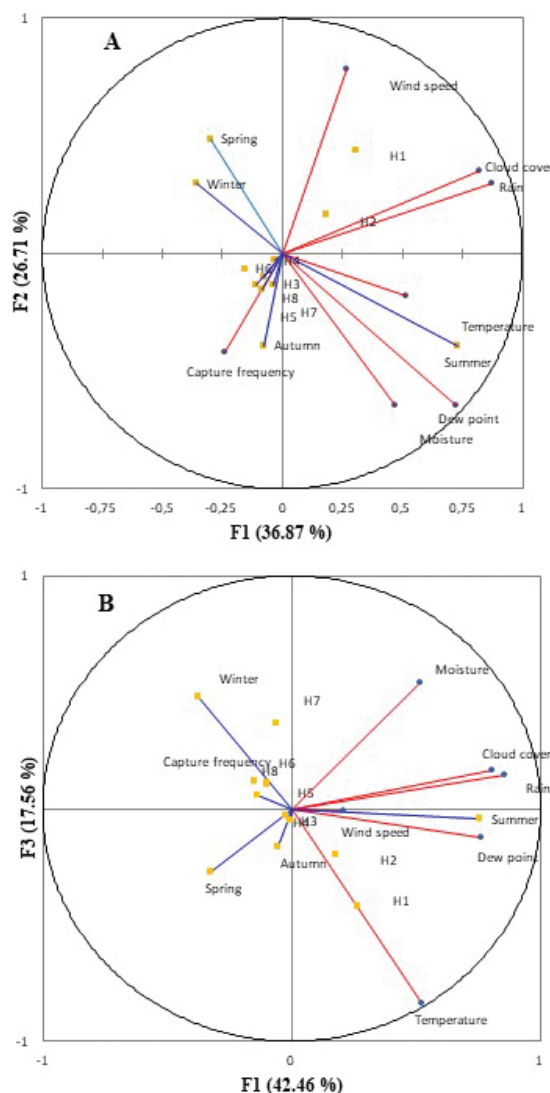


Figure 3. Ordination Biplot of the Principal Correspondence Analysis applied on the environmental parameters measured in each season for studied time slots of *Porcellio albinus* locomotor activity. H1 (16 h–18 h); H2 (18 h–20 h); H3 (20 h–22 h); H4 (22 h–00 h); H5 (00 h–2 h); H6 (2 h–4 h); H7 (4 h–6 h); H8 (6 h–8 h).

respectively positive and negative. The positive correlation between temperature and summer and the negative correlation between temperature and winter are probably important to explain the difference observed in capture frequency between winter and summer.

Discussion

The desert species *P. albinus* survives extreme heat and drought in desert regions thanks to the microclimate within the burrows they dig, analogous to the behaviour of *H. reaumurii* (Medini-Bouaziz 2018; Linsenmair 1975; Hoffmann 1983). The burrows are dug according to standards that consider the prevailing wind direction, the geo-environmental conditions, and the type of vegetation covering the nebkas (Medini-Bouaziz et al. 2017a). During the night, larger specimens (> 8 mm in body length) emerge from their burrow to forage (Medini-Bouaziz et al. 2017b). The absence of specimens less than 8 mm outside burrows could be explained by parental care during the first two months of the life of juveniles which is a sensitive period for the species' survival (Medini-Bouaziz et al. 2017b). Specimens of *P. albinus* were active every night except during rainy nights. Despite its scarcity in desert environments, rainfall can play an important role in regulating the activity of *P. albinus*, which remains inside the burrow for days after rain falls and shows no activity outside its shelter until the sand is completely dry (Medini-Bouaziz 2018). This could be explained by the fact that *P. albinus*, when leaving its burrow for foraging, piles up sand from the burrow in front of the opening (Linsenmair 2007). This sand, marked by the owner's individual chemical signature, is then used as a landmark to find its burrow back when returning (Linsenmair 2007; Medini-Bouaziz et al. 2017a).

In *P. albinus*, there must be an innate 24-hour rhythm controlled by the rhythmic and natural variations of the duration of the dark period since animals only emerge from their burrows during the dark period. *Porcellio albinus* exhibits a different activity rhythm compared to *H. reaumurii*, although the two species are from similar guild of burrowing species and occupy the same habitat in Zarat. *Hemilepistus reaumurii* shows diurnal surface activity (Cloudsley-Thompson 1956a) from February to November and remains inactive during December and January (Ayari et al. 2018). Nocturnal activity as observed in *P. albinus* is conventional for the majority of terrestrial isopods, while diurnal activity of *Hemilepistus* is an evolutionary novelty. The reason for the shift to daytime activity in *H. reaumurii* could be to avoid competition with other burrowing species such as *P. albinus* (Jaramillo et al. 2003), but also to avoid predation pressure by scorpions.

A nocturnal and photonegative lifestyle could be explained by the species' water balance. *Porcellio albinus* is a drought-sensitive species with a high rate of water loss through transpiration (Cloudsley-Thompson 1956b). The reverse is true for *H. reaumurii*, a species with a low transpiration rate and a thick cuticle (Ayari et al. 2016). Thus, the degree of nocturnal activity of terrestrial isopods is correlated with the ability to withstand water loss by transpiration.

Interseasonal comparisons of capture frequencies show that the epigeic activity of *P. albinus* in Zarat is high at the beginning and the end of the dark period in winter and summer, respectively. This nocturnal peak is more spread out in spring and autumn and occurs in the middle of the night. In general, most of the variability recorded in daily activity patterns in terrestrial isopod activity could be explained by habitat type and season. Tuf and J rabkova (2008) found different activity patterns across seasons and between closed forests and clear-cut areas. Terrestrial isopods are known to cope with differing environmental conditions in their habitat by adopting various patterns of activity in response to these conditions (Warburg et al. 1984). Among littoral isopods, nocturnal species such as the genus *Ligia* or *Tylos europeus* Arcangeli, 1938 (Colombini et al. 1996) prefer high humidity (Warburg et al. 1984; Jaramillo et al. 2003). Terrestrial isopods from mesic habitats (such as the genera *Oniscus*, *Porcellio*, and *Armadillidium*) responded less strong to light and humidity compared to littoral species.

Although the number of different forms of activity, and the quantitative relationships between these forms are correlated with air humidity (see, e.g., Den Boer (1961) for *Porcellio scaber* Latreille, 1804), in xeric habitats, all isopods are positively hydrokinetic and negatively photoreactive (except for *H. reaumurii*) with a drop in activity at high temperatures (Warburg 1968). However, temperature and humidity were not the only factors affecting surface locomotor activity of *P. albinus* and the interseasonal variability observed could also be explained by wind speed negatively affecting the capture frequency. Similar results have been recorded in *Oniscus asellus* Linnaeus, 1758 and *Porcellio scaber* where the number of wandering isopods on the surface decreased with increasing wind speed (Cloudsley-Thompson and Gupta 1960). Of course, environmental variables such as wind speed could also indirectly influence temperature and humidity.

In conclusion, *P. albinus* is a nocturnal species showing activity patterns depending upon temperature, humidity, and related environmental variables. These activity patterns also strongly depend on the season with a strong peak in activity during the early night in winter and towards the end of the night in summer when temperatures and humidity are most favourable while activity is more evenly distributed throughout the night during spring and autumn.

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