Life history, cell size and physiological performance of the common rough woodlouse (*Porcellio scaber*)
(Crustacea: Malacostraca: Isopoda)

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Summary

Land colonization was one of the most important steps in life evolution. Many taxa colonized land, and among Crustaceans the most successful colonizers were isopods – the group which settled on land during the second wave of land invasion and subsequently adapted to a wide range of terrestrial habitats. Addressing biological characteristics that potentially relate to the capacity of isopods to colonize and inhabit terrestrial environments, I studied (study I-IV) different elements of the life history and thermal biology of the common rough woodlouse (*Porcellio scaber*), a common species of terrestrial isopods. The characteristics taken into account included offspring size and clutch size, growth pattern, the size of respiratory organs, cell size and physiological performance. Apart from offspring size and clutch size, all other traits were studied with reference to their reactions to either prolonged (lifetime) or acute (minutes, hours) changes in temperature and oxygen conditions. Ultimately, my research aimed at creating a coherent view of an isopod, as a life history strategy that maximizes fitness under challenges imposed by the environmental variance.

My first study (study I) explored effects of offspring brooding on the origin of (i) an indeterminate growth strategy and (ii) a positive correlation between female size and offspring size. To achieve this goal, I measured offspring size, clutch size and female size in *P. scaber* and evaluated how much *P. scaber* females grow after maturation. To formulate general conclusions about isopods, I reviewed published data on different isopod species, testing how common ly isopods evolved a positive correlation between offspring size and female size. My next two studies of *P. scaber* (study II & III) focused on the effects of temperature and oxygen supply on (i) woodlouse growth with reference to the size of respiratory organs (pleopodal lungs) (study II), and (ii) woodlouse performance under metabolically demanding conditions with reference to cell size in eyes and the gut (study III). Both studies took advantage of a long term experiment in which *P. scaber* woodlice were reared from egg to adulthood in two oxygen concentrations (10 and 22 % O₂) and two temperatures (15 and 22 °C). My fourth study (study IV) examined effects of oxygen concentration on the thermal performance of *P. scaber*, which involved four behavioral experiments measuring the effects of oxygen availability on preferred temperatures, thermal dependence of locomotor activity, and the maximal and minimal critical temperatures.

Study I revealed a positive correlation between the body mass of *P. scaber* females and the body mass of their offspring, but this relationship existed only among females with extremely small clutches. According to my literature review, this life history pattern was very rarely studied in isopods, but available data suggest that it might characterize 50% of isopod species. According to life history models, brooding may have consequences for
the investments of females to offspring, resulting in a positive correlation between offspring size and female size. Indeterminate growth was reported previously in isopods and this growth pattern was also confirmed by my results for *P. scaber*. The smallest reproducing female weighted 21.682 mg and the biggest weighted 131.236 mg, which means that the majority of growth is realized in *P. scaber* females after maturation. According to life history models, this type of growth strategy might evolve as a consequence of costs and limits imposed by brooding. **Study II** revealed that *P. scaber* woodlice grew faster in high temperatures and oxygen availability did not affect their growth. Also, *P. scaber* developed smaller lungs in response to either higher temperatures or lower oxygen availability (with the exception of adult females). This result suggests that the irresponsiveness of growth pattern of *P. scaber* to oxygen conditions may be related to plastic changes in lung size. **Study III** demonstrated that the cells of *P. scaber* in the eyes and the gut were slightly smaller under low oxygen availability, but their size was not affected by temperature (smaller cells are proposed to be beneficial at higher temperatures and low oxygen availability). Conditions in which animals were reared did not affect their ability to cope with metabolically demanding conditions. **Study IV** demonstrated that *P. scaber* exposed to low oxygen availability preferred lower temperatures, run slower, achieved the maximal speed in lower temperature and had lower maximal critical temperatures than animals in normal atmospheric oxygen concentration. These results support the idea of oxygen limitation of thermal tolerance and performance and also indicate possible ecological consequences of differences in oxygen availability in natural habitats of isopods.

Although my research mainly concerned *P. scaber*, it offers an opportunity to formulate three generalizations about isopods, which have apparent relevance for isopods’ ecology: (i) brooding may be an important evolutionary factor for isopods that leads to the origin of a positive correlation between offspring size and mother size and favors indeterminate growth strategy; (ii) thermal and oxygen conditions during development should be regarded as two important environmental factors that shape isopods’ traits, with temperature affecting mainly lung size and somatic growth, and oxygen level affecting mainly lung size and to some extent cell size; (iii) acute changes of oxygen conditions should be regarded as an important factor that shapes isopods’ thermal performance and thermal tolerance as well as their preferences for thermal conditions in microhabitats.
Streszczenie

Kolonizacja lądu była jednym z ważniejszych wydarzeń w ewolucji życia na Ziemi. Formy lądowe występują w wielu taksonach i na przykład spośród skorupiaków najefektywniej ląd skolonizowały przedstawiciele rzędu równonogów (Isopoda). Równonogi zasiedliły ląd w czasie drugiej fali kolonizacji i w wyniku ewolucji przystosowały się do życia w wielu różnorodnych środowiskach. W celu bliższego poznania cech potencjalnie związanych ze zdolnością równonogów do skolonizowania i zasiedlenia różnorodnych środowisk lądowych zbałem (Badania I-IV) różne składowe historii życiowej i biologii termalnej prosionka szorstkiego (Porcellio scaber) – pospolitego gatunku równonoga lądowego. Cechy, które wziąłem pod uwagę to m.in.: liczebność miotu i wielkość pojedynczego osobnika potomnego, krzywa wzrostu, rozmiar organów wymiany gazowej, wielkość komórek i wydolność fizjologiczna. Prócz problemu kształtowania wielkości potomstwa i liczebności miotu wszystkie inne cechy badałem w kontekście ich reakcji na długotrwałe (całe życie) lub gwałtowne (minuty, godziny) zmiany w temperaturze i zawartości tlenu w powietrzu. Ostatecznie moje badania miały na celu wytworzenie spójnego modelu równonoga jako strategii życiowej, która maksymalizuje dostosowanie pod presją tworzoną przez zmienne środowisko życia.

Mój pierwszy projekt badawczy (Badania I) miał na celu zbadanie wpływu opieki rodzicielskiej (rozwój potomstwa w strukturach ciała matki) na ewolucję (i) niezdeterminowanego wzrostu oraz (ii) pozytywnej korelacji między wielkością matki a wielkością jej potomstwa. W tym celu zmierzyłem średnią wielkość osobnika potomnego, liczebność miotu i wielkość matki u prosionka szorstkiego oraz oszacowałem możliwość wzrostu samic po osiągnięciu dojrzalości. Żeby sformułować bardziej ogólne wnioski dotyczące całego rzędu równonogów dokonałem przeglądu literatury dotyczącej rozrodu równonogów, którego celem było sprawdzenie jak powszechne jest występowanie pozytywnej korelacji między wielkością matki i wielkością potomstwa w tej grupie. Kolejne dwa projekty badawcze (Badania II i III) koncentrowały się na wpływie temperatury i dostępności tlenu na (i) wzrost prosionków z odniesieniem do wielkości narządów wymiany gazowej (płuca pleopodalne) (Badania II) oraz (ii) osiągi fizjologiczne prosionków w wymagających metabolicznie warunkach w odniesieniu do wielkości ich komórek w oku i jelitach (Badania III). Obydwa projekty badawcze bazowały na długoterminowym eksperymencie, w którym prosionki były hodowane od stadium jaja do dorosłości w dwóch stężeniach tlenu (10 and 22 % O₂) i dwóch temperaturach (15 and 22 °C). Czwarty projekt badawczy (Badania IV) miał na celu sprawdzenie wpływu zawartości tlenu na osiągi fizjologiczne w gradiencie temperatury. W ramach tego projektu przeprowadziłem cztery
eksperymenty behawioralne, w których mierzyłem wpływ obniżonej zawartości tlenu na preferowaną temperaturę, zależność między temperaturą a aktywnością lokomotoryczną oraz na minimalne i maksymalne krytyczne temperatury.

Badania I pokazały pozytywną korelację między masą ciała samic prosionka szorstkiego i masą ciała potomstwa, ale jedynie w przypadku bardzo mało liczebnych miotów. Z przeglądu literatury wynika, że zależność taka była rzadko badana u równonógów, ale dotychczasowe dane sugerują, że u 50% przebadanych gatunków korelacja wielkości matki z wielkością potomstwa jest pozytywna. Zgodnie z modelami teoretycznymi oznacza to, że rozwój młodych w komorze lęgowej u równonógów może mieć ewolucyjne konsekwencje w postaci inwastycji matki w pojedyncze potomstwo co w rezultacie może prowadzić do ewolucji pozytywnej korelacji między masą ciała matki a masą ciała jej potomstwa. Wzrost niezdeterminowany był wcześniej opisywany u równonógów, a moje badania potwierdzają jego występowanie u P. scaber. Najmniejsza rozmnażająca się samica P. scaber ważyła 21.682 mg, a największa 131.236 mg co pokazuje, że u tego gatunku większa część wzrostu zachodzi po osiągnięciu dojrzałości. Taka strategia wzrostu może wyewoluować (zgodnie z teoretycznymi modelami historii życiowych) jako konsekwencja kosztów i ograniczeń wynikających z rozwój potomstwa w komorach lęgowych.

Badania II pokazały, że w wysokich temperaturach prosionki rosły szybciej, a dostępność tlenu nie wpływała na ich wzrost. Jednocześnie, w wysokiej temperaturze płuc było mniejsze i taki sam efekt był obecny u zwierząt rozwijających się w niskiej zawartości tlenu (za wyjątkiem dorosłych samic). Wynik ten sugeruje, że brak reakcji na zawartość tlenu na poziomie tempa wzrostu może być związany z plastycznymi zmianami wielkości płuc.

Badania III pokazały, że komórki prosionka szorstkiego były nieznacznie mniejsze w warunkach niskiej zawartości tlenu (posiadanie małych komórek powinno być korzystniejsze w wysokiej temperaturze i niskiej zawartości tlenu). Temperatura i zawartość tlenu w jakich zwierzęta się rozwijały nie miały wpływu na ich zdolność do radzenia sobie z wymagającymi metabolicznym warunkami. Badania IV pokazały, że prosionki pod wpływem obniżonej zawartości tlenu wybierały mikrośrodowiska o niższej temperaturze, poruszaly się wolniej, osiągały maksymalną prędkość poruszania się w niższej temperaturze oraz miały niższą maksymalną temperaturę krytyczną niż zwierzęta badane w warunkach normalnej zawartości tlenu. Te wyniki wspierają hipotezy przewidywające, że zarówno tolerancja termiczna jak i wydolność fizjologiczna organizmów zmienności w różnych temperaturach może być kształtowana przez dostęp do tlenu. Wyniki te wskazują także na możliwe ekologiczne konsekwencje zróżnicowania warunków tlenowych w środowisku życia równonógów.
Chociaż moje badania koncentrowały się na jednym gatunku (prosionku szorstkim) to dają one możliwość sformułowania trzech uogólnionych wniosków mających znaczenie dla zrozumienia ekologii wszystkich równonogów. (i) Rozwój potomstwa w komorze lęgowej samicy może być czynnikiem prowadzącym do wyewoluowania pozytywnej zależności między wielkością matki a wielkością jej potomstwa. (ii) Termiczne i tlenowe warunki czasie rozwoju powinny być traktowane jako dwa istotne czynniki środowiskowe kształtujące różne cechy u równonogów. Temperatura wpływa głównie na wielkość płuc i tempo wzrostu a tlen wpływa na wielkość płuc i w małym stopniu na wielkość komórek. (iii) Gwałtowne i krótkotrwałe zmiany w warunkach tlenowych powinny być traktowane jako istotny czynnik, który kształtuje tolerancję wysokich temperatur u równonogów oraz ich krzywą osiągów w gradiencie temperatury. Również ma wpływ na wybór warunków termicznych dokonywany przez równonog w ich mikrosiedliskach.
### General introduction

Land colonization was one of the most dramatic steps in the history of life on Earth and it occurred independently several times. The first big wave of animals’ land colonization took place about 490 Mya at the break of Cambrian and Ordovician periods. The second wave happened ca. 300 Mya in Carboniferous and coincided with the peak of atmospheric oxygen concentration (up to 35%, which would now be considered hiperoxia), which was then followed by a drastic drop in oxygen concentration (to even 10%, which would now be considered hypoxia) (Berner et al., 2007). It means that animals which colonized land with the second wave must have been dealing not only with higher insolation, desiccation, higher thermal variance than their aquatic ancestors but also they faced a decreasing oxygen level (because air oxygen dissolves in water, changes in the atmospheric oxygen were also likely affecting oxygen conditions in the aquatic environments). One of the groups that were evolving a terrestrial life style during the second wave of land colonization were isopods (Broly et al., 2013). As far as fossils tell, in early Cretaceous isopods already occupied terrestrial habitats spanning from Western Europe to Eastern Asia and they already were well adapted to land (Broly et al., 2013), thus the key terrestrial adaptations of isopods must had been evolving during the phase of decreasing oxygen concentration in the atmosphere of Earth.

Extant terrestrial isopods occupy a wide range of terrestrial habitats from semi-aquatic intertidal zones to extremely dry deserts and they are regarded as the best land adapted group of crustaceans (Hornung, 2011). It is important to notice that isopods have been exposed to varying level of oxygen conditions not only in the geological timescale (evolution under decreasing oxygen concentration), but also in the spatial scale, e.g. they occupy a broad range of elevations, reaching even up to 4725 m a.s.l. (Beron, 2008). What is more, oxygen conditions can vary across isopods’ habitats with a level of leaf litter decomposition rates or fluctuate through time e.g. in temporarily flooded burrows (Wright and Ting, 2006).

Considering the dramatic history of land colonization by isopods, I studied life history and physiological traits of the common rough woodlouse (*Porcellio scaber*), a terrestrial isopod species that originates from Europe (excluding its SE part), but now is widespread also in North America and Australia (Schmalfuss, 2003). My ultimate aim was to gain an insight into characteristics of traits that were evolving among isopods upon their colonization of terrestrial environments. I focused especially on the patterns of growth and investment to offspring production as well as the size of cells in different organs and the size of gas-exchange organs, connecting these traits to woodlice performance along gradients of thermal and oxygen conditions in the environment. Cell size might be the character shaping
the performance of animals according to environmental conditions like temperature, oxygen and food deprivation. I envisioned that in a larger perspective differences in performance may have consequences for longevity and tissue production potential, which should have effect on resource allocation and finally on the evolution of basic life history traits like growth rate, offspring size.

To address detailed hypotheses inspired by theories considering life history, cell size and physiological performance I conducted four studies of *P. scaber*. The reports from these studies in a form of published papers or submitted manuscripts form my thesis (hereafter, studies I-IV).

**Life history strategy**

A life history of an organism can be described by its “decisions” concerning allocation of resources to growth, reproduction, maintenance, storage and other activities over a lifetime. In consequence, a life history strategy defines the management of limited resources in order to maximize fitness. The main characteristics of a life history strategy are: longevity, mortality, survivability, reproductive characteristics, growth curve shape, an investment in offspring size vs. number and a moment of switching from growth to reproduction. The life history approach describes an organism as it is “seen” by selection, which is the most adequate view of the biology of an organism in a wide evolutionary context (Kozłowski, 1992; Stearns, 1992).

Isopods as well as many crustaceans engage in a costly parental (maternal) care in the form of marsupial brooding, which is an important element of their life history strategy. On the ventral body side females form a brood pouch built from exoskeleton in which they lay eggs and where eggs and early juveniles stages undergo development. A brooding isopod female provides developing offspring with nutrients and oxygen (Hoese and Janssen, 1989; Lardies et al., 2004) and maintains aqueous environment during the first stages of marsupial development. Although costs of parental care in isopods have not been thoroughly investigated, there is some evidence that brooding females might be handicapped by mobility (Kight and Ozga, 2001). Because of the importance of brooding for fitness maximization, I have become interested in its effects on isopods’ life history traits, especially offspring size and growth pattern (as also suggested by theoretical works of Heino and Kaitala (1996) and Jørgensen et al. (2011)).

Jørgensen et al. (2011) used life history modeling to show that offspring brooding can select larger females to increase offspring provisioning at the costs of offspring number, leading to a positive correlation between maternal size and offspring size. Such pattern has been reported in some species of arthropods (Fox and Czesak, 2000) and fish (Hendry et al.,
but generally it has been rarely studied. According to Jørgensen and colleagues, evolution of increasing offspring size with maternal size can be caused by a tight temporary link between the future of offspring and that of a female. This reasoning considers the importance of size dependent mortality and decreased survivability during brooding. Under such scenario, higher survivability of bigger mothers can lead to a prolonged brooding period and changes in optimal offspring size. In consequence, bigger mothers can provide better parental care and produce bigger offspring.

If offspring brooding is associated with costs (either decreased physiological performance or increased mortality) for a parent, another life history pattern can evolve, namely an indeterminate growth strategy (Heino and Kaitala, 1996). Indeterminate growth is a type of growth when animals do not cease growth after maturation but continue to enlarge body size throughout the entire life. Classical life history models predict that this mode of growth is adaptive only in a seasonal environment (Kozłowski, 2006; Stearns, 1992), but according to Heino and Kaitala (1996), brooding may increase adaptive value of indeterminate growth also in a non-seasonal environment. Indeterminate growth is observed in many isopod species, which continue to molt and grow for the entire life, in contrast to insects which typically cease growth at the imago stage.

The size of animals can be determined not only by maternal investment. Also environmental factors can play a role here and one of these factors is temperature. How temperature affects growth and adult size is an interesting and important question. A few patterns can be observed here. One of the most widely known and discussed is an evolutionary puzzle called Temperature Size Rule (TSR; Atkinson, 1994). It bases on the repeatable observations that in low temperature ectothermic animals grow slower, but reach bigger final body sizes compared to conspecifics developing in high temperatures. One of the possible factors which can explain this puzzle is an effect of oxygen. Indeed, in some studies the TSR was demonstrated in animals developing under hypoxic conditions (Hoefnagel and Verberk, 2015; Walczyńska et al., 2015).

To test the hypotheses inspired by life history theory, I conducted a study which involved collecting correlative data on *P. scaber* from the field, and then integrated this information with a similar type of published data for different species of isopods (study I). I also studied growth pattern of isopods reared in two temperatures and two oxygen levels in a long term experiment (study II). The study of growth pattern also considered plastic changes in the size of gas-exchange organs, and I predicted that a potential for this plasticity can reduce effects of rearing conditions on growth pattern.
The question about how cell size contributes to body size changes and whether this contribution affects Darwinian fitness has been addressed by researchers since 1950s (Davison, 1956) but it was more developed by Szarski (1983). In his theoretical work, Szarski proposed a distinction between life history strategies that are characterized either by frugal or wasteful physiologies. A frugal strategy would be characterized by “slow life rate”, slow metabolism, slow locomotion, not well developed neural system and bigger cells, with a wasteful strategy being the opposite. Further developments of these ideas resulted in the Theory of Optimal Cell Size (TOCS; Czarnoleski et al., 2017, 2013; Kozłowski et al., 2003), which considers that the size of cells in a body is a subject of selection, depending on environmental conditions that differentiate metabolic supply and oxygen demand. From this perspective, oxygen and temperature conditions in the environment might be regarded as two main selective drivers when addressing adaptive changes in cell size. On one hand, a small cell has a big relative area of membranes, enabling an organism to maintain high respiration rate, which would be beneficial in high temperatures where metabolic needs of ectothermic animals remain high (Czarnoleski et al., 2017). There is also evidence to show that oxygen transportation is more efficient in cell membranes than in aqueous cytosol (Subczyński et al., 1989), therefore a small cell should be also more efficient under hypoxia because their big relative area of membranes should speed oxygen delivery to mitochondria (Czarnoleski et al., 2013). On the other hand, the large area of membranes incurs costs because cells maintain ion gradients on their surface, which requires resources and ATP. These costs might be reflected in a variance of mass-specific metabolic rates as organisms of the same body size but built of cells of different size should differ in respiration rate. Indeed, various research has demonstrated this phenomenon (Chown et al., 2007; Czarnoleski et al., 2018; Hermaniuk et al., 2017; Maciak et al., 2014; Starostová et al., 2013, 2009).

Isopods seem to be an excellent group for addressing predictions of TOCS because they are naturally challenged by different oxygen and thermal conditions and they continue growth for the entire life. We do not know to what extent isopods are able to plastically change their cell size in response to living conditions. Nevertheless, this type of plasticity evolved in other groups like flies (Czarnoleski et al., 2013) or lizards (Czarnoleski et al., 2017), so we have a good reason to expect this plasticity also in isopods, predicting that it helps isopods to adjust their physiology and performance to different developmental conditions. To test these predictions, I measured cell size in isopods originating form a long term experimental study, reared in different temperature and oxygen levels, and then examined metabolic capacities of isopods with different cell sizes (study III).
Physiological performance

In the introductory paragraphs, I have stressed that isopods are challenged by different oxygen conditions in natural habitats and they were also challenged by varying oxygen availability during the course of evolution. An important question is to what extent isopods are able to tolerate oxygen deficiency and what consequences this deficiency may have on their physiological performance. Measuring performance gives important biological information only if a measured character is directly linked to animals’ fitness and can therefore be considered to have an adaptive value. To measure environmental effects on performance, a bunch of different approximations can be used, such as consumption rate (Gudowska and Bauchinger, 2018), metabolic rate (Wright and Ting, 2006), mobility (Dailey et al., 2009; Schuler et al., 2011) or growth (Hoefnagel and Verberk, 2015). Performance of ectotherms is often measured in a gradient of body temperature, which results in the so-called thermal performance curve. The shape of a thermal performance curve is typically asymmetrical, indicating a slow increase of performance with increasing temperature towards some maximum, followed by a rapid decrease in performance with a further increase in temperature (Angiletta, 2009). It is important to stress here that the maximum of a given performance is not necessarily a real optimal temperature from the perspective of fitness maximization (in fact, this important distinction seems to be often overlooked by studies of thermal biology of ectotherms, but see Clark et al. (2013)). For instance, if an animal is given a possibility to choose thermal environments, a chosen temperature may not be the same as the temperature in which the animal achieves the highest performance (e.g. moves at the highest speed). For this reason, it is valuable to evaluate preferred temperatures together with temperatures that maximize different aspects of performance.

A thermal performance curve has two points in which it reaches “zero performance”. These temperatures can be described as critical temperatures in which an organism stops its “normal” functioning. These values can be obtained from a thermal performance curve (by interpolation), but they can be biased by computational artifacts. For this reason, it seems reasonable to obtain a performance curve and thermal extrema (minimal and maximal critical temperatures) independently, conducting separate experiments. Thermal performance of ectotherms can be shaped by oxygen supply, which is visible especially in higher temperatures where demand for oxygen resulting from increasing metabolic rate is likely to be in a mismatch with oxygen supply resulting from deteriorated oxygen transportation, e.g. caused by circulatory system, lower affinity of carrying proteins and changes in membrane state (Verberk et al., 2016). According to the Oxygen and Capacity Limited Thermal Tolerance Theory (OCLTT; Pörtner, 2001), this mismatch might be the main driver of thermal limits in ectotherms, acting before protein denaturation caused by increased
temperatures. To test the effects of hypoxia on thermal performance, I ran four behavioral experiments on *P. scaber* exposed to different thermal and oxygen conditions (study IV).

**Study I**

My first study addressed the question if extended parental care of isopods in a form of their marsupial brooding is associated with a positive correlation between offspring body size and female body size and with an indeterminate growth pattern. I tested this hypothesis in two complementary steps. In the first step, I obtained gravid females of common rough woodlouse from the wild and after parturition under laboratory conditions, I collected information on the number of offspring and offspring body size in each brood. In the second step, I performed an extensive review of published works on different species of isopods, collecting species-specific information on offspring numbers and offspring size and the relation of these two traits with female size. I hypothesized that because of parental care, a positive relation between offspring size and mother size would prevail among isopods, including my study species *P. scaber*. I also, considered that a strategy of indeterminate growth in *P. scaber* would be manifested by a wide range of body masses among brooding females.

**Studies II & III**

Studies II and III took advantage of a long term (2.5 years) developmental experiment on *P. scaber* (Horváthová et al., 2017, 2015). During this experiment, I reared woodlice from the egg to adult individuals in two different oxygen concentrations (10% and 22% O\textsubscript{2}) and two temperatures (15°C and 22°C). During the course of this experiment, animals were measured and dissected in regular intervals to provide information for growth pattern and the size of gas-exchange organs. At the end of the experiment, respiration rate of mature animals was measured, which was followed by dissections of animals that provided microscopic slides for cell size measurements.

In **Study II**, I checked how development in different oxygen and temperature conditions affected the size of respiratory organs (pleopodal lungs) of the studied woodlice. I also tested whether woodlice rearing conditions affected growth pattern according to Temperature Size Rule (larger size in colder environment). For this purpose, I used data collected from developing as well as adult woodlice (analyzed separately). I expected that development under low oxygen level or high temperature should lead to bigger lung size, considering that this response would provide sufficient oxygen supply under demanding conditions (warm environment combined with hypoxia). To explore if differences in lung size might affect life history, I analyzed growth pattern of woodlice, predicting that if lung size
undergoes changes with oxygen conditions, then this response would prevent growth pattern to differentiate between the experimental treatments.

In Study III, I tested predictions of the theory of optimal cell size (TOCS) in mature animals. For this purpose I performed measurements of the respiration rate of woodlice under two extreme conditions (22 °C combined with 10% O₂ – the most demanding conditions, and 15 °C combined with 22% O₂ – the least demanding conditions). After these measurements, animals were dissected to measure cell size in three different organs. I measured the size of ommatidia (this element of an eye is built from a constant number of nine cells, therefore its size was used as a proxy of cell size), the size of hepatopancreas B cells and hindgut epithelial cells. I hypothesized that animals developed in low oxygen level and high temperature would have smaller cell size and thus an increased capacity to cope with demanding metabolic conditions. For animals originating from low temperature and high oxygen level I expected a reverse pattern.

Study IV

In this part of my work, I checked whether oxygen conditions affect thermal performance of P. scaber. For this purpose, I ran four complementary experiments on P. scaber and each experiment was testing different thermal phenomena. In Experiment I, I tested how low oxygen supply affected preferred temperature, exposing woodlice to a thermal gradient under two atmospheric oxygen concentrations. The experimental animals were able to freely choose a site where they wanted to stay. In Experiment II, I checked how hypoxia affected running speed of woodlice in different temperatures, forcing animals to run in a range of temperatures under two different oxygen conditions. In Experiments III & IV, I checked how hypoxia affected maximal and minimal critical temperature, measuring at which temperature the animals were losing their performance. In my experiments, I generally predicted that hypoxia would cause woodlice to prefer cooler sites, reduce their locomotor performance and shift the performance maximum to lower temperatures. Also, I expected that the upper thermal limits of woodlice would be shifted down by hypoxia, but I did not expect effects of hypoxia for the lower thermal limits, as suggested by previous studies of other ectotherms (Klok et al., 2004; Stevens et al., 2010).
STUDY I

Size dependence of offspring production in isopods: a synthesis
Size dependence of offspring production in isopods: a synthesis

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Abstract

In isopods, parental care takes the form of offspring brooding in marsupial pouches. Marsupial brooding was an important step towards the origin of terrestrial lifestyles among isopods, but its potential role in shaping isopod life histories remains unknown. It is here considered that marsupial brooding imposes costs and creates a temporary association between the survival of mothers and that of their offspring. Integrating findings from different life history models, we predicted that the effects of marsupial brooding set selective conditions for the continuation of growth after maturation, which leads to indeterminate growth, and the production of larger offspring by larger females. Based on this perspective, a study on the size dependence of offspring production in the woodlouse Porcellio scaber was performed and the generality of the results was tested by reviewing the literature on offspring production in other isopods. In P. scaber and almost all the other studied isopods, clutch size is positively related to female size. Such dependence is a necessary pre-condition for the evolution of indeterminate growth. The body mass of P. scaber differed six-fold between the largest and smallest brooding females, indicating a high potential for post-maturation growth. Our review showed that offspring size is a rarely studied trait in isopods and that it correlates negatively with offspring number but positively with female size in nearly half of the studied species. Our study of P. scaber revealed similar patterns, but the positive effect of female size on offspring size occurred only in smaller broods, and the negative relation between clutch size and offspring size occurred only in larger females. We conclude that the intraspecific patterns of offspring production in isopods agree with theoretical predictions regarding the role of offspring brooding in shaping the adaptive patterns of female investment in growth, reproduction, and the parental care provided to individual offspring.
Keywords
clutch size, female size, indeterminate growth, life history evolution, offspring brooding, offspring size, parental care, trade-off

Introduction
Most crustaceans engage in different types of parental care, which, in isopods, takes the form of offspring brooding in marsupia (Thiel 2000, Vogt 2016). During the moult preceding reproduction, the isopod female produces exoskeletal extrusions that form a marsupial pouch, which is used as a chamber for egg laying and carrying developing larvae (Hornung 2011, Appel et al. 2011). At the end of brooding, the female releases the offspring into the environment. In terrestrial species, individuals inside the marsupium undergo the change from the aqueous to the gaseous environment (Horváthová et al. 2015). Marsupial brooding was crucial for the origin of terrestrial lifestyles in isopods (Hornung 2011, Appel et al. 2011, Horváthová et al. 2017). Interestingly, land colonisation occurred independently at least twice in the evolutionary history of this group (Lins et al. 2017). Here, we consider that marsupial brooding plays a role in the evolution of life history strategies, especially by affecting adaptive patterns of female investment in growth, reproduction, and the parental care provided to individual offspring. To the best of our knowledge, this perspective remains largely unexplored in isopods.

The theory of life history evolution predicts that resource availability limits imposed by physiological and ecological circumstances forces organisms to optimise the lifetime allocation of investment among growth, reproduction and other competing demands to ensure the highest expected fitness under given mortality and production conditions (Stearns 1992). Adopting this basic principle, life history modelling has demonstrated that somatic growth is beneficial as long as one calorie invested in increasing body mass increases the future expected reproductive output by more than one calorie (Kozłowski 1992). Likewise, organisms are expected to optimise the amount of resources retained over unfavourable periods to fuel activities in favourable periods (Ejsmond et al. 2015); the timing of reproductive activity during a season, compromising the future prospects of offspring (Ejsmond et al. 2010); and the amount of resources invested in single offspring, compromising offspring number (Smith and Fretwell 1974). Developments in life history theory have led to an important conclusion: there is a wealth of distant optima with similar fitness consequences, which explains why life histories are so enormously diverse in nature (Stearns 1992, Czarnoleski et al. 2003, Kozłowski 2006).

A range of life history models predict the evolution of a bang-bang resource allocation strategy, which is associated with the complete cessation of growth after maturation and the so-called determinate growth pattern (Kozłowski and Wieger 1987, Stearns 1992, Kozłowski 2006). In contrast, many isopods continue to moult after maturation, combining their capacity for reproduction with the capacity for somatic
growth. This ability results in the potential for continuation of growth for the entire life span and the so-called indeterminate growth pattern. Beside isopods and some other crustaceans, indeterminate growth has evolved in annelids, molluscs, fish, amphibians, and reptiles (Kozłowski 1996). The prevalence of indeterminate growth in nature awaits explanation, but life history theory predicts that this growth strategy provides fitness advantages if the capacity to produce new tissue and/or survive strongly increases with body mass and if these capacities change discontinuously through time (Stearns 1992, Perrin and Sibly 1993, Kozłowski 2006). Modelling of optimal allocation has shown that discontinuities driven by either seasonal changes in mortality/productive capacity (Kozłowski and Teriokhin 1999) or unequal future prospects of offspring released into the environment at different times of the year (Ejsmond et al. 2010) lead to the evolution of alternating shifts between the investment in somatic growth and that in reproduction, resulting in the indeterminate growth pattern. For many organisms, including isopods, seasonality is the primary selective force responsible for the evolution of indeterminate growth. Nevertheless, specific characteristics of species biology, such as the reproduction via clutches instead of via a series of single offspring, can elicit discontinuous changes in mortality/production capacity, similar in principle to the effects of seasonality (Czarnoleski and Kozłowski 1998). Such characteristics can help explain why indeterminate growth originated among annuals or perennials living in non-seasonal environments. Heino and Kaitala (1996) designed a life history model for gill-brooding unionid mussels (e.g., Sinanodonta woodiana Labecka and Domagala 2018) and demonstrated that an indeterminate growth pattern can evolve in non-seasonal environments if carrying the offspring is associated with costs, either decreased physiological performance or increased mortality and with a temporary association between the fate of the offspring and the survival of the parent. Importantly, using a different model to explore the role of parental care in the evolution of offspring size among fish, Jørgensen et al. (2011) concluded that offspring brooding selects for the increased investment of larger females in individual offspring. For indeterminately growing animals, such a strategy involves constant changes in the optimal size of offspring as females increase their body mass. Under this strategy, the production of larger offspring is expected to require prolonged brooding, which temporarily links the fate of the offspring with that of the mother. If larger females have higher survival probability than do smaller females, then the increased investment in individual offspring becomes more beneficial for larger females. Overall, these theoretical considerations suggest that marsupial brooding might be an important driver of growth strategy and offspring size in isopods. To investigate this hypothesis, we performed a study on the common rough woodlouse (Porcellio scaber) and evaluated the generality of our results by analysing data from the literature on other isopods. We aimed at integrating information on intraspecific patterns of size dependence in offspring production over as wide a range of isopod species as possible. In particular, we focused on the relationships between female size and the number and size of offspring in broods and on evidence of an allocation trade-off between the number and size of the offspring in broods. Generally, we expected reproductive capacity to increase as females grow in size, which is the
fundamental condition favouring the strategy of indeterminate growth (see above). Therefore, we expected a positive relationship between female body size and clutch mass/clutch size (hypothesis i). We also tested this relationship for non-linearity, assuming that a negative allometry would indicate an increased relative space limitation in larger females, whereas a positive allometry would indicate a decreased relative space limitation in larger females. Next, we examined whether the investment of females in individual offspring increased with the size of females, which should produce a positive correlation between the average offspring mass in a brood and female body mass (hypothesis ii). Finally, we analysed data on the mean mass of offspring in relation to the number of offspring per brood, looking for an allocation trade-off between offspring size and number (hypothesis iii).

Materials and methods
A case study of *Porcellio scaber*

In June–July 2014, individuals of *P. scaber* were collected in an old backyard in Kraków, Poland. In our study, we used females in the 3rd and 4th stages of brood development (classified according to Lardies et al. 2004a). Each gravid female was placed in a plastic box (100 ml). The boxes were perforated to provide aeration, lined with paper towel and supplied with a piece of moist sponge (water source), a piece of clay pot (shelter) and the dry leaves of the alder (*Alnus glutinosa*) and ash (*Fraxinus excelsior*), which served as *ad libitum* food source. For additional control of humidity, the boxes were placed inside a larger plastic container with wet sand in the bottom. The container with boxes was placed in a shaded patio of the Institute of Environmental Sciences, Jagiellonian University in Kraków. Each day, the boxes were assessed for the presence of new offspring. Emerging offspring were collected, and the female was weighed to the nearest 0.001 mg (Mettler Toledo XP26, Greifensee, Switzerland). The clutches were dried for one hour at 60 °C in an oven (UFE 400, Memmert GmbH + Co. KG, Germany), and the dry mass of each clutch was measured to the nearest 0.001 mg (Mettler Toledo XP26, Greifensee). The offspring in each clutch were counted under a stereoscopic microscope. To calculate the mean dry mass of a single offspring, we divided the clutch dry mass by the number of offspring.

All statistical analyses were performed with R 3.4.1 software (R Core Team 2017), and the rgl package of R (Adler and Duncan Murdoch 2017) was used to create graphs. To test whether larger females produced heavier and larger clutches (hypothesis i) and larger offspring (hypothesis ii), we correlated clutch dry mass, clutch size, and mean offspring dry mass with female body mass. To evaluate the nature of these relations, we fitted linear and power regression models to our data and selected the best model using AIC. In this way, we did not a priori assume any particular relationship between the studied variables. When fitting our regression functions, we used either an ordinary least square (OLS) method or the weighted least square (WLS) method, which
allowed us to account for the observed increase in the variance of dependent variables at higher values of an independent variable. Note that the OLS method assumes homogeneity in the variances of the independent variables. According to Knaub (2009), the issue of non-homogeneity can be overcome by using the WLS method, which assigns decreasing weights to observations with increasing levels of variance. Following Knaub’s (2009) procedure, we first ordered our data according to an increasing value of an independent variable to identify four quartiles. For data from the first quartile, the weights were calculated as an inverse of the highest value of the independent variable in this quartile (56.328 mg). For data from the other quartiles, the weights were calculated as the inverse of the actual value of the independent variable. To examine whether larger offspring emerged from smaller clutches (hypothesis iii), we used a multiple regression analysis with the mean offspring mass as a dependent variable and clutch size and female body mass as two independent variables. The use of a multiple regression allowed us to dissect the independent effects of each of the two independent variables. Thus, we also re-examined hypothesis (ii) regarding the link between female size and offspring size, with a control for the potential links between clutch size and offspring size. We allowed our model to consider an interaction between our two independent variables. Therefore, to assess the independent effects of each variable (partial regression), we estimated and tested this effect after centring the whole model in either the minimum or maximum value of each independent variable (Quinn and Keough 2002). The multiple regression analysis was performed with the use of either OLS or WLS, and the best model was chosen based on AIC.

**Intraspecific patterns in isopods**

To evaluate the generality of our hypotheses (i–iii) and the empirical results for *P. scaber*, we reviewed the published literature on isopods for intraspecific information on at least one of the following relationships: clutch size with female size, offspring size with female size, and clutch size with offspring size. Relevant publications were identified by an extensive search of keywords in scientific databases, the review of reference lists of available publications and by personal communication with specialists in the field. Whenever we found relevant information regarding one of the three relationships, we classified the relationship as either statistically significant or non-significant; we also identified significant relationships as either positive or negative. If available, correlation coefficient (r) values were also assigned to each relationship. Traits used to study the relationship between female size and either clutch size or mass varied substantially among authors and species; therefore, we additionally recorded information regarding the types of measured traits. For each type of relationship, each species was classified according to the nature of this relationship, integrating all the results on a species reported in the literature. If a relationship for a given species was consistently reported to be significantly positive, significantly negative, or non-significant, the species was regarded as exhibiting a positive (+) or negative (-) relationship or no relationship (NS).
Species for which mixed results were reported, showing either non-significant/significantly positive relationships or non-significant/significantly negative relationships were classified as NS/+ or NS/−, respectively. Ultimately, we used this integrated species information to calculate how frequently among the studied isopods a given pattern (+, −, NS, NS/+ and NS/−) of each relationship occurred. In addition, we used a 1-4 scale to evaluate the confidence in the support for each pattern (+, −, NS, NS/+ and NS/−) to predict the directions of the studied relationships (hypotheses i-iii). Consistently positive/negative relationships (+/−) were treated as providing reliable evidence to support or oppose a hypothesis. Non-significant patterns (NS) were regarded as not supporting a hypothesis, but we also considered the possibility that they might represent false negatives due to low statistical power. The level of support given by inconsistent results (NS/+ and NS/−) was dependent on the context. If among the non-significant and significant results, the significant results were consistent with our predictions, we treated the mixed results as weakly supporting our hypothesis. However, if the significant results were in conflict with the predictions, we regarded the mixed results as strongly opposing the hypothesis.

Results

A case study of Porcellio scaber

Among 101 brooding females of P. scaber, body mass ranged from 21.682 to 131.236 mg, clutch sizes ranged from 7 to 106 juveniles, and the mean dry body mass of offspring ranged from 0.078 to 0.126 mg between clutches. Larger females produced heavier (r = 0.83, t_{1,99} = 14.9, p<0.001, Fig. 1A) and larger clutches (r = 0.83, t_{1,99} = 15.09, p<0.001, Fig. 1B), but the mean offspring mass did not show a consistent relationship with female mass (r = 0.14, t_{1,99} = 1.44, p = 0.15, Fig. 1C). Comparison of AIC between the alternative regression models showed that a linear weighted regression produced the best fit to our data (Fig. 1). Therefore, we concluded that clutch size and clutch mass increased linearly with female body mass, which is consistent with our finding that the dry body mass of offspring did not change systematically with female body mass.

The results of the multiple regression analysis (Fig. 2) showed no effect of clutch size (t_{1,97} = 0.74, p = 0.46) and a positive effect of female mass (t_{1,97} = 2.38, p = 0.02) on the mean dry body mass of offspring. The interaction between the two independent variables was non-significant (t_{1,97} = -1.60, p = 0.11). When we centred the model at the value of the smallest broods (7 offspring), the positive link between offspring dry mass and female body mass was still significant (t_{1,97} = 2.39, p = 0.02), but the significance disappeared when we centred the model at the value of the largest clutches (107 offspring) (t_{1,97} = -0.22, p = 0.83). When we centred the model at the minimum female body mass (21.682 mg.), clutch size and offspring body mass appeared to be unrelated (t_{1,97} = 0.44, p=0.66), but centring at the maximum body mass (131.236 mg) revealed
Figure 1. In *Porcellio scaber*, the dry mass of clutches (A) and clutch size (B) increased linearly with female body mass, but the mean dry mass of offspring did not depend on female mass in a consistent way (C). Lines represent fitted regressions

A: $y = -0.13 + 0.08x$ ($r = 0.83$, $p<0.001$)

B: $y = -0.32 + 0.74x$ ($r = 0.83$, $p<0.001$)

C: $y = 0.1 + 0.00006x$ ($r = 0.14$, $p = 0.15$).
In Porcellio scaber, the heaviest offspring were released by large females that produced small clutches. The plane represents a multiple regression model fitted to the data; the partial slopes depicted on the edges were calculated by setting the other predictor value to its minimum and maximum values. A negative relationship between clutch size and offspring body mass, though the effect was marginally significant ($t_{1,97}=-1.98, p=0.05$). Overall, this analysis indicated that the largest offspring were produced by large females with small clutches.

**Intraspecific patterns in isopods**

Our literature search identified a total of 79 species of isopods that were studied with respect to at least one of the following relationships: clutch size with female size (Fig. 3A), offspring size with female size (Fig. 3B), and clutch size with offspring size (Fig. 3C). Detailed results of the review are provided in Table 1S (Suppl. material 1). The effect of female size on clutch size was the most frequently studied relationship (79 species), while the relationships between female size and offspring size and between offspring size and clutch size were studied in only 18 and 7 species, respectively, including *P. scaber* as reported in this study. For the vast majority of the studied isopods (Fig. 3A), we found evidence that supports a positive relationship between female size and clutch size (hypothesis i). Importantly, we found no reports of the opposite pattern and only
Figure 3. The literature search identified 79 species of isopods that were studied with respect to at least one of the following relationships: clutch size with female size (A), offspring size with female size (B), and clutch size with offspring size (C). Each graph shows how frequently a given nature of each relationship was found among the studied isopod species. The exact number of species for which the relationships A, B, C were evaluated is given by N. For each type of the relationships A, B, C each species was classified according to the nature of this relationship. If a relationship for a given species was consistently reported to be significantly positive, negative, or non-significant, the species was marked by a positive (+) or negative (−) symbol or by NS. Species for which mixed results were reported in the literature, showing either non-significant/significantly positive relationships or non-significant/significantly negative relationships, were marked by NS/+ or NS/−, respectively. Colour intensity indicates values along a 1–4 scale of confidence to the support provided by each relationship pattern (+, −, NS, NS/+ and NS/−) to hypotheses (i–iii). Relationship A: a positive relationship predicted between female body size and clutch mass/clutch size (hypothesis i). Relationship B: a positive correlation predicted between the average offspring mass in a brood and female body mass (hypothesis ii). Relationship C: a negative correlation predicted between the mean mass of offspring and the number of offspring per brood (hypothesis iii).

occasional reports of a non-significant pattern. However, the non-significant reports were typically found along with reports of significantly positive patterns, suggesting that many of the non-significant results might be false negatives. For nearly half of the species (Fig. 3B, C), we found evidence that supports a positive relationship between female size and offspring size (hypothesis ii) and a trade-off between offspring size and clutch size (hypothesis iii).
Discussion

Growth patterns vary considerably in nature (Stearns 1992, Czarnoleski et al. 2003, 2005, Ejsmond et al. 2010), but understanding the origin of this variance is more challenging than it might initially appear. Our data on *P. scaber* suggest that this species of woodlouse has evolved a life history strategy with intense resource allocation to somatic growth in the reproductively mature stages. We found up to six-fold differences in body mass between the largest and the smallest brooding females, which suggests that only 20% of the body mass of a fully-grown female might be achieved before maturation, with the majority of growth potentially occurring with reproduction in such instances. Consistent with the idea that species with indeterminate growth should be characterized by a strong dependence of reproductive capacity on body size (hypothesis i), we found that larger females of *P. scaber* carried larger and heavier broods. This evidence clearly shows that mature females can gain reproductive capacity by further increasing body mass. The results of our literature search indicate that such size dependence is widespread among other isopod species. Interestingly, we found no reports of a negative pattern of this relationship and few reports of non-significant effects of female size on clutch size, which are likely to be false negatives. A strong size dependence of reproductive capacity promotes the evolution of iteroparous breeding with indeterminate growth, but alone, it is not sufficient to explain such evolution (Heino and Kaitala 1996, Czarnoleski and Kozłowski 1998). In fact, some isopods, such as *Ligia oceanica*, have evolved a semelparous breeding strategy with determinate growth, despite the size dependence of reproductive capacity (Sutton et al. 1984, Willows 1987). Given this, what might be the ultimate drivers of the evolution of indeterminate growth in isopods? A life history theory calls attention to the pattern of resource allocation among growth, reproduction and other competing demands, which should be optimised to ensure the highest expected lifetime fitness in given mortality and production conditions (Stearns 1992, Kozłowski 2006). Considering this idea, the alternating allocations between growth and reproduction that lead to indeterminate growth reflect changes in allocation optima, with temporal shifts in the capacity to survive and/or reproduce. The woodlouse *P. scaber* and many other isopod species inhabit seasonal environments, and life history models have demonstrated that seasonal alternations of the periods suitable for survival, offspring production, and growth with less favourable periods establish the selective forces that favour the continuation of somatic growth after maturation (Kozłowski and Teriokhin 1996, Czarnoleski and Kozłowski 1998, Ejsmond et al. 2010). However, as suggested by Heino and Kaitala (1996) and Czarnoleski and Kozłowski (1998), the strategy of indeterminate growth might also bring additional fitness benefits if organisms engage in offspring brooding. Carrying offspring creates temporary changes in mortality/physiological performance and links between the fate of the offspring and that of the mother, leading to shifts in the optimality of growth and reproduction through time. Unfortunately, the costs associated with offspring brooding are poorly studied in isopods, but we might expect them in the form of increased vulnerability to predation and/or increased energetic costs associated with locomotion and supplementation of offspring. For example, Kight and Ozga (2001) observed that gravid...
females of Porcellio laevis were less mobile than were non-gravid females. In addition, female isopods are postulated to regulate the pH and osmolality of their marsupial fluids and provision their broods with necessary resources via the so-called cotyledon (Lardies et al. 2004a). Furthermore, Lardies et al. (2004a) showed that gravid females had lower ingestion rates and digestibility and higher metabolic rates than did non-gravid ones. Interestingly, Perrin and Sibly (1993) suggested another mechanism that favours indeterminate growth among offspring brooders, which is non-exclusive of the hypothesis of a role of discontinuities in mortality/physiological capacity. If current offspring production is limited by the space provided by the brooding cavities rather than by the physiological capacity to produce new tissue, organisms are selected to direct surplus resources to further somatic growth, thereby increasing their fertility at the following reproductive event. There is some evidence to suggest that the maximal reproductive performance of isopods might be restricted by the volume of the marsupial pouches (Lardies et al. 2004a, Appel et al. 2011). Nevertheless, we found no indication that such limitations change with body size in females of P. scaber. The relationship between clutch size and female size did not deviate from linearity. In addition, we detected substantial variance in the mass of clutches produced by females of a given body size, which suggests that reproductive capacity might not be entirely dependent on the space limitation of the marsupium, unless the volume of the marsupium is highly variable at a given body mass.

Our data on P. scaber show that the dry body mass of offspring differed between broods by as much as 62%. A significant part of this variance was linked to differences in clutch size and female body mass, but the pattern of this dependence was complex. Supporting hypothesis ii, the size of offspring was positively related to female size, but this pattern existed only if we considered small clutches. Focusing on larger clutches, we found no apparent relationship between offspring size and female size. In accord with hypothesis iii, the size and number of offspring were inversely related, but this pattern existed only among larger females. In broods produced by smaller females, the two traits were not correlated. To date, studies of isopods have only occasionally addressed the question of whether offspring size changes with either female size or clutch size. According to our literature search, the relationships between female size and offspring size and between offspring size and clutch size have only been studied in 18 and 7 species, respectively. For nearly half of these species, we found evidence that supports a positive relationship between female size and offspring size (hypothesis ii) and a trade-off between offspring size and clutch size (hypothesis iii). It is suggestive that all studies that failed to find evidence of such a trade-off (Fig. 3C) overlooked the potential effects of female size in the statistical analysis of offspring size and clutch size data. In effect, many of these results might represent false negatives because differences in clutch size driven by female size are not primarily generated by the trade-off between offspring size and number but rather by the higher capacity of larger animals to produce new tissue (as shown in Figs 1A, 3A). Furthermore, the positive effects of female size on offspring size can lead to a positive correlation between offspring size and clutch size. Apparently, this is the case in the isopod Bethalus pretoriensis (Telford and Dangerfield 1995), which was the only species we found for which a positive association
between clutch size and offspring size was reported; furthermore, a positive association between offspring size and female size was found in this species (see Suppl. material 1).

Examples of life history strategies in which offspring size is a function of parent size are rare in nature, and their evolutionary origins are puzzling (Rollinson and Rowe 2016). Apart from isopods, positive relationships between offspring size and female size have previously been reported in some other arthropods (Fox and Czesak 2000) and some species of snakes (Ford and Seigel 2011) and fish (Hendry et al. 2001, Hendry and Day 2003). Interestingly, in the pipefish (Syngnathidae), the positive relationship between offspring size and female size characterized pouch-brooding species but not ventral-brooding species (Braga Goncalves et al. 2011). In isopods, the positive correlation between female size and offspring size was also demonstrated on the interspecific level (Sutton et al. 1984). Different phenomena have been invoked to understand why larger females might produce larger offspring, including competition between siblings (Parker and Begon 1986), unequal benefits from increased fecundity in small vs large females (McGinley 1989), varying efficiency of resource acquisition from parents (Sakai and Harada 2001), increased parental mortality during reproduction (Kindsvater and Otto 2014), and an increased capacity of larger females to meet the overhead costs of reproduction (Filin 2015). With different degrees of relevance, each of these phenomena might apply to isopods. Nevertheless, here we consider that in live-bearing organisms such as isopods, the survival of offspring during brooding is tightly linked to the survival of the parent, a concept that has helped explain the evolution of indeterminate growth pattern in isopods. According to the life history model of Jørgensen et al. (2011), this tight association promotes increased investment in individual offspring by larger females if larger females have improved survival compared to smaller females. If the development of larger offspring requires longer brooding and if brooding is costly, then the production of larger offspring should be more beneficial to larger females because brooding is relatively less costly for them. Importantly, this scenario can help to rationalise the complex pattern found in our data on *P. scaber*. It is suggestive that larger females produced larger offspring only if we considered small broods. We can expect that a small brood (several offspring in our case) is relatively more costly for small females than for large females, which have much higher reproductive potential (more than 100 offspring in our case). If the cost of brooding corresponds to the risk of mortality, then larger brooding females with small broods should suffer relatively lower costs, which should select them for increased investment in individual offspring. Certainly, before drawing firm conclusions regarding this phenomenon, future studies should better identify how the costs of marsupial brooding change with clutch size and female size.

Conclusions

Based on the integrated findings reported here, we can attempt to form conclusions about the most common patterns in the size dependence of isopod reproduction and
the significance of these patterns for understanding the evolution of isopod life histories. In nearly all the studied species, we found a strong size dependence of female reproductive capacity. Such a dependence is important for explaining the evolution of an indeterminate growth strategy in many species of isopods. Data from nearly half of the isopod species revealed a negative relationship between offspring size and offspring number and a positive relationship between mother size and offspring size. Importantly, our case study of P. scaber suggests that the emergence of each pattern is context-dependent: a positive effect of female size on offspring size was observed only in smaller broods, and a negative relationship between clutch size and offspring size was observed only for larger females. We propose that these patterns be viewed as different elements of a single phenomenon: a lifetime strategy of investment in growth, reproduction and the parental care provided to single offspring that is shaped by selective conditions. The key message of this study is that to gain a better understanding of this strategy in isopods, we must consider the effects of marsupial brooding, especially its costs and the linkage between the survival of mothers and that of their offspring. We hope that our synthesis of theoretical ideas and data on isopods will increase the intersection of life history theory and empirical research in isopods and that this work will stimulate further theory development and lead to an improved understanding of the ecology and evolution of isopods.

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References


Supplementary material 1

Table S1
Authors: Andrzej Antol, Marcin Czarnoleski
Data type: species data
Explanation note: Results of the literature search for reports of at least one of the following relationships in isopods: clutch size vs female size, offspring size vs female size, offspring size vs clutch size.
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Link: https://doi.org/10.3897/zookeys.801.23677.suppl1
Supplementary material

Table 1

Results of the literature search for reports of at least one of the following relationships in isopods: clutch size vs female size, offspring size vs female size, offspring size vs clutch size.

The “Habitat” column labels each species according to its typical lifestyle: aquatic or terrestrial, or aquatic/terrestrial if it occupies an intertidal zone. The “Effect” column indicates whether a given relation was positively/negatively significant (+ or -) or nonsignificant (NS). If available, the values of the correlation coefficients (r) are reported next to each relation.

The offspring size vs clutch size relationship was studied in two ways, both with and without simultaneous consideration of the effect of female size. The analyses that considered female size focused on a trade-off between allocation to clutch size and to offspring size. To differentiate between these two non-equivalent results, the results obtained with consideration of the effects of female size are marked by asterisks (*). The trade-off present at the interpopulation level is marked with two asterisks (**). The “Size measures” column shows what size measures were used to study the relationship between clutch size and female size:
e.n. – egg/embryo number, m.n. – manca number, b.l. – body length, b.m. – body mass (w.e. – with eggs), b.w. – body width, c.l. – cephalic length, c.w. – cephalothorax width, h.w. – head width, b.a. – body area, log – logarithmic transformation of data, cube – cubic transformation of data.

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<td>+ 0.62</td>
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<td>Great Britain</td>
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<td>+</td>
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<td>Japan</td>
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<td>Great Britain</td>
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<td>NS, Tunisia</td>
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<td>+</td>
<td>Israel</td>
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<td>e.n. b.m.</td>
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<td>0.84</td>
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<tr>
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<td>+</td>
<td>0.95</td>
<td>e.n. b.l.</td>
<td>Morocco [4]</td>
<td></td>
</tr>
<tr>
<td><em>Porcellio dalensis</em></td>
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<td>+</td>
<td>0.95</td>
<td>m.n. b.l.</td>
<td>Morocco [4]</td>
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<td><em>Porcellio ficulneus</em></td>
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<td>NS</td>
<td>0.25</td>
<td>m.n. b.m.</td>
<td>Israel [51]</td>
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<tr>
<td><em>Porcellio laevis</em></td>
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<td>+</td>
<td>0.62</td>
<td>e.n. b.l.</td>
<td>Chile [57]</td>
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<td>0.86</td>
<td>e.n. b.l.</td>
<td>Chile [57]</td>
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<td>+</td>
<td>0.94</td>
<td>e.n. b.m.w.e.</td>
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<td>0.95</td>
<td>e.n. b.l.</td>
<td>Morocco [4]</td>
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<td>+</td>
<td>0.95</td>
<td>m.n. b.l.</td>
<td>Morocco [4]</td>
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<tr>
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<td>+</td>
<td></td>
<td>e.n. b.l.</td>
<td>Tunisia [58]</td>
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</tr>
<tr>
<td><em>Porcellio lamellatus</em></td>
<td>terrestrial</td>
<td>+</td>
<td></td>
<td>e.n. b.l.</td>
<td>Tunisia [58]</td>
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</tr>
<tr>
<td><em>Porcellio olivieri</em></td>
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<td>+</td>
<td></td>
<td>e.n. b.m.</td>
<td>Israel [55]</td>
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<tr>
<td><em>Porcellio scaber</em></td>
<td>terrestrial</td>
<td>+</td>
<td>0.83</td>
<td>log m.n. log b.m.</td>
<td>NS 0.27 NS* 0.12</td>
<td>Poland this study</td>
</tr>
<tr>
<td><em>Porcellio scaber</em></td>
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<td>+</td>
<td>0.83</td>
<td>log m.n. log b.m.</td>
<td>+ 0.6 -. -0.54</td>
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</tr>
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<td>0.83</td>
<td>log m.n. log b.m.</td>
<td>NS 0.1 NS 0.02</td>
<td>Poland this study</td>
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<td></td>
<td>m.n. b.m.</td>
<td>Poland [60]</td>
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<tr>
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<td>0.82</td>
<td>e.n. b.l.</td>
<td>Libya [44]</td>
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<tr>
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<td>+</td>
<td>0.69</td>
<td>e.n. b.l.</td>
<td>Japan [12]</td>
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<td>+</td>
<td>0.75</td>
<td>log e.n. log b.m.</td>
<td>NS 0</td>
<td>Pennsylvania [7]</td>
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<tr>
<td><em>Porcellio siculoccidentalis</em></td>
<td>terrestrial</td>
<td>+</td>
<td>0.92</td>
<td>e.n. b.l.</td>
<td>Italy [45]</td>
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</tr>
<tr>
<td><em>Porcellio siculoccidentalis</em></td>
<td>terrestrial</td>
<td>+</td>
<td>0.97</td>
<td>e.n. b.l.</td>
<td>Italy [45]</td>
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</tr>
<tr>
<td><em>Porcellio siculoccidentalis</em></td>
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<td>+</td>
<td>0.92</td>
<td>m.n. c.l.</td>
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<td>0.62</td>
<td>e.n. b.l.</td>
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<td>0.82</td>
<td>e.n. b.l.</td>
<td>Tunisia [46]</td>
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<td><em>Porcellionides sexfasciatus</em></td>
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<td>+</td>
<td>0.95</td>
<td>e.n. b.l.</td>
<td>Morocco [4]</td>
<td></td>
</tr>
<tr>
<td><em>Porcellionides sexfasciatus</em></td>
<td>terrestrial</td>
<td>+</td>
<td>0.95</td>
<td>m.n. b.l.</td>
<td>Morocco [4]</td>
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<td>Location 1</td>
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<tr>
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<td>+ 0.25</td>
<td>m.n.</td>
<td>b.m.</td>
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<td>b.l.</td>
<td>Tunisia</td>
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<td>m.n.</td>
<td>b.l.</td>
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<td>e.n.</td>
<td>b.l.</td>
<td>Morocco</td>
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</tr>
<tr>
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<td>m.n.</td>
<td>b.l.</td>
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<td>NS 0.1</td>
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<td>NS -0.2</td>
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<td>Botswana</td>
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<td>m.n.</td>
<td>b.m.</td>
<td>Zimbabwe</td>
<td>[62]</td>
</tr>
<tr>
<td>Porcellionides pruinosus</td>
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<td>+ 0.82</td>
<td>e.n.</td>
<td>b.l.</td>
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<td>Schizidium tiberianum</td>
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<td>+ 0.77</td>
<td>m.n.</td>
<td>b.m.</td>
<td>Israel</td>
<td>[51]</td>
</tr>
<tr>
<td>Serolis cornuta</td>
<td>aquatic</td>
<td>+</td>
<td>e.n.</td>
<td>b.l.</td>
<td>Antarctic</td>
<td>[47]</td>
</tr>
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<td>Serolis cornuta</td>
<td>aquatic</td>
<td>+</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Weddell Sea</td>
<td>[48]</td>
</tr>
<tr>
<td>Serolis cornuta</td>
<td>aquatic</td>
<td>+</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Weddell Sea</td>
<td>[48]</td>
</tr>
<tr>
<td>Serolis cornuta</td>
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<td>+</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Signy Island</td>
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<td>+ 0.73</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Antarctic</td>
<td>[49]</td>
</tr>
<tr>
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<td>+ 0.81</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Antarctic</td>
<td>[49]</td>
</tr>
<tr>
<td>Serolis polita</td>
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<td>+ 0.41</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Antarctic</td>
<td>[49]</td>
</tr>
<tr>
<td>Serolis septemcarinata</td>
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<td>+ 0.67</td>
<td>e.n.</td>
<td>b.w.</td>
<td>Antarctic</td>
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<tr>
<td>Soteriscus gaditamus</td>
<td>terrestrial</td>
<td>+ 0.89</td>
<td>e.n.</td>
<td>b.l.</td>
<td>Morocco</td>
<td>[4]</td>
</tr>
<tr>
<td>Soteriscus gaditamus</td>
<td>terrestrial</td>
<td>+ 0.89</td>
<td>m.n.</td>
<td>b.l.</td>
<td>Morocco</td>
<td>[4]</td>
</tr>
<tr>
<td>Sphaeroma serratum</td>
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<td>NS 0.02</td>
<td>e.n.</td>
<td>b.l.</td>
<td>Tunisia</td>
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<td>+ 0.85</td>
<td>e.n.</td>
<td>b.l.</td>
<td>Romania</td>
<td>[40]</td>
</tr>
<tr>
<td>Trachelipus nodulosus</td>
<td>terrestrial</td>
<td>+</td>
<td>e.n.</td>
<td>h.w.</td>
<td>Hungary</td>
<td>[53]</td>
</tr>
<tr>
<td>Trachelipus rathkii</td>
<td>terrestrial</td>
<td>+ 0.87</td>
<td>log e.n.</td>
<td>log b.m</td>
<td>NS 0.06</td>
<td>Pennsylvania</td>
</tr>
<tr>
<td>Trachelipus rathkii</td>
<td>terrestrial</td>
<td>+ 0.73</td>
<td>log e.n.</td>
<td>log b.m</td>
<td>- -0.5</td>
<td>Pennsylvania</td>
</tr>
<tr>
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STUDY II

Thermal and oxygen conditions during development cause common rough woodlice (*Porcellio scaber*) to alter the size of their gas-exchange organs

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Thermal and oxygen conditions during development cause common rough woodlice (*Porcellio scaber*) to alter the size of their gas-exchange organs.

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Abstract

Terrestrial isopods have evolved pleopodal lungs that provide access to the rich aerial supply of oxygen. However, isopods occupy conditions with wide thermal and oxygen gradients, suggesting that they might have evolved adaptive developmental plasticity in their respiratory organs to help meet metabolic demand over a wide range of oxygen conditions.

To explore this plasticity, we conducted an experiment in which we reared common rough woodlice (*Porcellio scaber*) from eggs to maturation at different temperatures (15 and 22°C) combined with different oxygen levels (10% and 22% O₂). We sampled animals during development (only females) and then examined mature adults (both sexes). We compared
woodlice between treatments with respect to the area of their pleopod exopodites (our proxy of lung size) and the shape of von Bertalanffy’s equations (our proxy of growth curves). Generally, males exhibited larger lungs than females. Woodlice also grew relatively fast but achieved a decreased asymptotic body mass in response to warm conditions; the oxygen level did not affect growth. Under hypoxic conditions, growing females developed larger lungs comparing to normoxia, but in only the late stage of development. Among mature animals, this effect was present in only males. Woodlice reared under warm conditions had relatively small lungs, and this effect was present in both developing females (the effect was increased in relatively large females) and among mature males and females.

Our results demonstrated that woodlice have evolved phenotypic plasticity in their lung size. We suggest that this plasticity helps woodlice equilibrate their gas exchange capacity to differences in the oxygen supply and metabolic demand along environmental temperature and oxygen gradients. The complex pattern of plasticity might indicate the effects of a balance between water conservation and oxygen uptake, which would be especially pronounced in mature females that need to generate an aqueous environment inside their brood pouch.

Key words
air breathing, gas exchange, hypoxia, isopods, land adaptation, respiratory organs

Introduction
Isopods (Arthropoda: Malacostraca: Isopoda) inhabit an extremely wide range of terrestrial and aquatic environments, including many extreme habitats such as intertidal zones (e.g., genera Ligia and Tylos; Warburg, 1987), caves (e.g., Mesoniscus graniger; Šustr et al., 2005), dry deserts (e.g., Hemilepistus spp.; Schmidt and Wägele, 2001), and either high elevations up to 4725 meters a.s.l. (e.g., Protracheoniscus nivalis; Beron, 2008) or oceanic depths down to 5500 meters (e.g., Munnopsidae family; Brandt et al., 2007). Isopods are also regarded as the most successful land colonizers among all crustaceans (Hornung, 2011), and the origin of terrestrial isopods is estimated to be approximately 300 Mya, between the Carboniferous and Permian periods (Broly et al., 2013). Aquatic ancestors of modern terrestrial isopods possessed characteristics such as brood pouches and mineralized cuticles, which likely facilitated the evolution a terrestrial life style (Hornung, 2011). Additionally, in the course of evolution, isopods gained specific adaptations to terrestrial environments, including lungs, a water conduction system, dermal tegumental glands, gut microbiota symbiosis or conglobation and aggregation behaviors (Cloudsley-Thompson, 1988; Hornung, 2011; Horváthová et al., 2017; Zimmer, 2002a).
The evolution of gas exchange structures, called pleopodal lungs, was apparently a key innovation among land-colonizing isopods (Csonka et al., 2013). Isopod lungs are formed by pleopod exopodites and are located on the ventral side of the body. Depending on the level of terrestrial specialization, different species of isopods have different lung structures (Hoese, 1982; Hsia et al., 2013; Schmidt and Wägele, 2001). Species less specialized for dry terrestrial environments have gas exchange surfaces directly exposed to the external environment. In drought-tolerant species, the gas exchange surface is located within lungs that are formed via invagination of the pleopod exopodite surfaces (Babula, 1981; Bielawski and Babula, 1980; Schmidt and Wägele, 2001) (Fig. 1 A, C). The lungs limit the direct contact of the respiratory organs with ambient air, which helps to conserve water (Hornung, 2011; Schmidt and Wägele, 2001). The lungs consist of a large number of branched tubules (pseudotracheae) formed by a thin respiratory epithelium (Bielawski and Babula, 1980; Wright and Ting, 2006). The apical side of the epithelial cells is covered with a cuticle (Bielawski and Babula, 1980). In relatively xeric isopod species, air enters the lungs through one or more openings called spiracles (Hoese, 1982; Hsia et al., 2013; Warburg, 1993). Interestingly, the cuticle that covers cells surrounding the spiracles can create microfolds (Fig. 1 A, B) that trap water; these humidify the entering air and serve as additional protection from drought (Babula, 1981). In insects with a tracheal system, oxygen diffuses directly into tissues and cells without mediation by oxygen-binding proteins (Klok et al., 2004; Wright and Ting, 2006). In contrast, terrestrial isopods possess a hemocyanin protein that binds oxygen in the lungs and then transports it via the hemolymph to the tissues (Klok et al., 2004; Wright and Ting, 2006).

Isopods that utilize ambient oxygen might be expected to be less oxygen-limited than aquatic species. However, even terrestrial isopods are likely challenged by poor oxygen (hypoxic) conditions, especially if they inhabit periodically flooded burrows, areas with high microbial respiration, such as decaying wood, habitats covered by snow, intertidal zones or simply high elevations (Harrison et al., 2018; Hoback and Stanley, 2001; Paim and Beckel, 1964; Wright and Ting, 2006). Like other ectotherms (Verberk et al., 2011), isopods also face a risk of an increasing incongruity between oxygen supply and demand with increasing temperatures. This incongruity was postulated to set thermal limits in ectotherms and might lead to functional hypoxia even in normoxic conditions (Harrison et al., 2018; Pörtner, 2001). To address the effects of this imbalance, we studied the common rough woodlouse (*Porcellio scaber*), a detritivorous terrestrial isopod, which inhabits leaf litter and compost, where it is likely to be challenged with hypoxic conditions (Wright and Ting, 2006). There is also evidence of *P. scaber* inhabiting locations with elevations up to 2100 meters a.s.l. (Beron,
2008). Originally, *P. scaber* woodlice occurred throughout Europe, but after spreading to other continents, the species is now distributed globally (Schmalfuss, 2003). Indirectly, such a wide geographic distribution suggests that *P. scaber* has high colonization potential and may adapt to a range of environmental conditions. The lungs of *P. scaber* represent the evolutionarily advanced type, characteristic of species well adapted to dry habitats, and they occur in two sexually dimorphic pairs of pleopods (Fig. 2 A–D).

Our study explored developmental changes in the lung size in *P. scaber* exposed to a gradient of thermal and oxygen conditions. We hypothesized that the increased demand for oxygen combined with an oxygen supply shortage will favor an increased gas exchange area. To address this hypothesis, we reared individuals of *P. scaber* from egg to an adult in four combinations of temperature and oxygen levels. If our hypothesis was correct, then poor access to oxygen, especially in combination with increased metabolic demands at high temperatures, would result in increased lung size. To assess links between changes in lung size and the life history strategy, we additionally estimated growth curves for the woodlice exposed to our treatments. If developmental plasticity in lung size helps to meet the metabolic demand for oxygen, then we expected no dramatic differences in the growth patterns in those exposed to different oxygen treatments.

**Materials and methods**

*Parental animals*

This work was a part of a long-term study on *P. scaber* previously described elsewhere (e.g., Horváthová et al., 2015a). In autumn 2013 and spring 2014, we collected specimens from an isolated population of *P. scaber* in an old backyard in Kraków, Poland (50°04’15.9"N 19°56’21.9"E) and transferred them to the Institute of Environmental Sciences (Jagiellonian University, Kraków). The field-collected animals served as the parents for a new generation, which was produced under controlled conditions and used in the laboratory experiments (see below). The field-collected animals underwent prolonged (weeks) acclimation to common laboratory conditions. The animals were fed *ad libitum* with a mixture of dried leaves of black alder (*Alnus glutinosa*) and European ash (*Fraxinus excelsior*) that were collected in a nearby forest. The females and males were maintained separately in plastic boxes that contained moist sand and pieces of broken clay pots as shelter. The animal boxes were maintained in temperature-controlled cabinet (Pol-Eko, Poland), with a 12 L:12 D photoperiod and a temperature of 15°C during the day and to 8°C at night, which simulated typical autumn/spring conditions in the Kraków area (short-day conditions). Acclimation was
the initial step in our procedure for stimulating synchronous offspring production among the parental generation (see below).

**Experimental setup**

The experiment was performed on a new generation of woodlice, which were obtained under controlled laboratory conditions. The experiment involved four developmental treatments created by combining two temperatures (15 and 22°C) with two oxygen concentrations (10% and 22%) in two thermostatic cabinets (Pol-Eko, Poland) set to either 15°C or 22°C and four 110-liter plexiglass chambers (40 × 50 × 55 cm; YETI, Agencja Reklamy, Kryspinów, Poland), with two chambers placed in each cabinet. One of the two chambers per cabinet was set to normoxic conditions (22%) and the other to hypoxic conditions (10%). Oxygen conditions inside the chambers were controlled by a four-channel ROXY-4 gas regulator (Sable Systems International, USA). The regulator was connected to gas tanks with nitrogen and oxygen (Air Products, Poland), supplying the normoxic chambers with oxygen (normoxia) and the hypoxic chambers with nitrogen. Inside the chambers, the air circulation was maintained with fans, and the relative humidity was controlled by four dew point generators DG-4 (Sable Systems International, USA), which were set to 75% relative humidity and connected to chambers via PP2 pumps (Sable Systems International, USA). The temperature and humidity inside the chambers were monitored with Hygrochron iButtons (Maxim/Dallas Semiconductor, USA). The relative humidity inside the boxes with the experimental animals reached 98%, which closely resembled conditions in the microhabitat occupied by the source population (Horváthová et al., 2015a).

To stimulate synchronous mating and egg production, the parental animals were allocated to 28 boxes for mating (40 males and 50 females in each box; in total, 1120 males and 1400 females), and the boxes were placed in the Plexiglas chambers that maintained the specific experimental conditions. This ensured that the new generation of woodlice experienced experimental conditions from the very beginning of their life cycle. Following McQueen and Steel (1980), the stimulation of offspring production involved exposing the short-day acclimated animals to a photoperiod with a prolonged light phase (16 L:8 D). Initially, all four experimental treatments involved descendants of the parental animals collected in autumn 2013, but due to the failure of one of our thermostatic cabinets, we lost some animals from the warm treatment. Given an expected developmental lag between cold- and warm-reared woodlice, we decided to continue the cold treatment and run the warm treatment again later, using descendants of the parental animals collected immediately after winter (spring 2014). There is no reason to believe that the parental animals give birth
to phenotypically different offspring in autumn vs spring, which would bias our comparisons of cold vs warm animals. All parental animals used in the experiment originated from the same population and represented the same generation/cohort. Importantly, the experiment was performed on a new generation of woodlice, which was produced under controlled laboratory conditions. Moreover, prior to the stimulation of mating in the laboratory, all parental animals experienced prolonged acclimation to the common laboratory environment and underwent a shift from short-day to long-day conditions, which should homogenize the phenotypic effects (if any) of prior environmental experience in the field-collected individuals.

The boxes with mating animals were checked weekly for the presence of gravid females. In isopods, egg laying and early juvenile development take place in a brood pouch called a marsupium, which develops on the ventral side of the female body during the parturial molt (a molt preceding reproduction) and is shed during the molt following reproduction. The offspring undergo twenty marsupial developmental stages (Milatović et al., 2010; Wolff, 2009). After leaving the marsupium, they go through two manca stages and twelve juvenile stages (Tomescu and Craciun, 1987; Zimmer, 2002b). In our experiment, gravid females were individually transferred to 100-ml plastic boxes with a layer of wet sand, a piece of a clay pot for shelter and dry leaves for food. The boxes with gravid females and subsequently the boxes with offspring were maintained under the same experimental conditions (in the same thermal cabinets with oxygen regulated chambers). The boxes were checked weekly, and if free-living mancae were observed, the female was removed from the box. The boxes with the offspring were sprayed weekly with water and supplemented with our standard mixture of tree leaves (see Animals section). During the first nine weeks of postmarsupial life, the offspring were fed with only leaves. Two-week-old juveniles were additionally fed one dead conspecific adult to provide them gut microbiota, which are important in this species for juvenile growth (Horváthová et al., 2015b). To provide enough protein, after the 4th week of life, the animals were fed one dried mealworm (Tenebrio molitor) once a week.

Individuals of the same sex from different clutches were pooled at the age of 20 weeks to form new single-sex groups, with 20 animals per group. Each group was placed in a separate box. When the females reached the body mass of mature woodlice in the wild (minimum 21.68 mg and average weight 76.83 mg following Antol and Czarnoleski (2018)), we added several males to each box to provide conditions for physiological maturation (Kight, 2008).
**Dissections and measurements**

To represent the respiratory capacity of *P. scaber*, we measured the areas of four flat-lying exopodites, and each woodlice was represented by the total area of exopodites (hereafter exopodite area), a proxy of lung size. According to Hoese (1982; Figs 5, 9) and Schmidt and Wägele (2001; Fig. 4), the pseudotracheal system occupies a significant portion of the exopodites in *P. scaber* and, compared to *Armadillo* and *Armadillidium* spp. (Isopoda), the exopodites of *P. scaber* have the most two-dimensional (flat) structure. To validate our proxy of lung size, we measured the areas of the whole exopodites and a dark area of these exopodites, which corresponds to a visible net of pseudotracheae (personal observations), and then we correlated the two areas. Note that the validation involved a subsample of 90 exopodites for which the dark areas with pseudotracheae were sufficiently visible for the measurements.

We started measuring the exopodites and body mass from the 5\textsuperscript{th} (22°C) and 8\textsuperscript{th} (15°C) months of postmarsupial life, when the animals reached a similar body size; the difference in time reflected the temperature-dependent difference in development. The measurements were conducted every two months over a 17-month period. The sex ratio was female biased, with approximately only 30\% males; therefore, we carried the measurements in only females that were randomly sampled from each experimental treatment. Additionally, at the end of the experiment, we sampled exopodites from individuals of both sexes (assumed to be fully mature animals). Each animal was weighed to the nearest 0.001 mg on a microbalance before dissection (Mettler Toledo XP 26, Mettler Toledo, Switzerland). During dissection, each individual was decapitated with a scalpel on a Petri dish. The body was submerged in 1x PBS (Avantor Performance Materials, Poland), and the exopodites were removed with forceps, placed in an Eppendorf-type tube and fixed in 10\% buffered neutral formalin (Krakchemia, Poland). Upon collection, samples were washed in 1x PBS, placed in a 1x PBS drop on a microscope slide and covered with a cover slip. Photos of the exopodites were taken in a bright field under a light microscope (Eclipse 80i, Nikon, Japan) equipped with a digital camera (Axio Cam MRC5, Zeiss, Germany) and image acquisition software (ZEN, Zeiss) under 4x objective magnification (Fig. 1). Photos were used to measure the areas of the exopodites as well as their dark areas containing pseudotracheae. Additionally, to visualize the structure of the exopodites, one pair of male and one pair of female formalin-fixed exopodites were prepared for scanning electron microscopy (SEM). After hydration, the exopodites were fixed in 1\% osmium tetroxide solution (Sigma-Aldrich Co., USA) in 1.6 \% potassium hexacyanoferrate (Chempur, Poland) for 2 hours at 4°C. Then, they were washed in distilled water, dehydrated in a series of ethanol concentrations and acetone,
dried in a CO₂ critical point drier (LADD, USA), sputter-coated with gold and viewed and photographed with a scanning electron microscope using 20 kV (HITACHI S-4700, Japan) in the Scanning Microscopy Laboratory at the Institute of Geological Sciences Jagiellonian University.

To measure the dark areas of the exopodites with pseudotracheae (validation of our proxy of lung size), we manually outlined the edges of the dark areas of the exopodites with ImageJ image analysis software (NIH, Bethesda, USA). The measurements of the areas of whole exopodites were performed with a multistep semiautomatic segmentation algorithm based on a Canny (1986) edge detector and morphological operations. More precisely, the successive steps of the algorithm were as follows: (i) load the RGB image; (ii) transform the image to grayscale image; (iii) downscale the image; (iv) blur the image; (v) apply the Canny edge detector; (vi) apply morphological dilation; (vii) fill the holes; (viii) apply morphological opening; (ix) apply morphological erosion; and (x) upscale the segmentation. The algorithm was implemented in MATLAB (The MathWorks, Inc., USA), which returned an image with coarse segmentation that was then corrected by the researcher. After correction, the software generated a CSV file with the area measurements in μm² as the final output. For each individual, we calculated the summed area of the four exopodites (exopodite area), our proxy of lung size, which served as a measure of the capacity for obtaining oxygen from the air. We excluded individuals for which we did not have a complete set of lung measures. Eventually, we obtained complete data from 334 growing individuals sampled over the course of the experiment and from 120 mature individuals sampled at the end of the experiment.

Statistical analysis

Statistical analyses were conducted in R software (R Core Team, 2018) with use of the effects (Fox and Weisberg, 2018) and ggplot2 (Wickham, 2016) packages, for mature isopods of both sexes and for the growing females sampled in the course of their development, respectively. To validate our proxy of lung size, we correlated the total of the dark areas of exopodites with pseudotracheae and the area of the whole exopodites. The analysis was performed separately for each pair of exopodites, while considering sex and developing woodlice vs adult woodlice. The data on our proxy of lung size (exopodite area) were analyzed with the general linear model with the exopodite area as a dependent variable, oxygen and temperature as fixed predictors, and body mass as a covariate. A similar model was applied for the mature animals, but it included sex as an additional fixed factor. We fitted the full models with all possible interactions and then applied a step-wise procedure that used the Akaike information criterion (AIC) to obtain the best model. If an interaction between
a covariate and categorical predictor was significant, we tested the significance of the main effects at the minimal, mean, and maximal values of the covariate. If the interaction of two (or more) categorical factors was significant, we analyzed differences between groups with a general linear hypothesis test using the multcomp package (Hothorn et al., 2008). Body mass and exopodite area were log transformed prior to the analysis.

To analyze links between growth and respiratory capacity, we used the nls function to fit von Bertalanffy growth curves (von Bertalanffy, 1957) to our body mass, $M_t$, data and the corresponding age, $t$, of animals. Although the mechanism proposed by Bertalanffy to explain the origin of indeterminate growth does not follow evolutionary principles, Bertalanffy’s mathematical formula can be used as a tool to describe the indeterminate growth pattern phenomenologically (Czarnoleski and Kozlowski, 1998). We used a three-parameter formula: 

$$M_t = M_A \left(1 - e^{-K(t-t_0)}\right)^3,$$

where $M_A$ is an asymptotic size, $K$ is a growth rate coefficient, and $t_0$ is the hypothetical age in which an animal would achieve a body size equal to 0. We estimated growth curve parameters and their confidence intervals separately for each treatment; the analysis involved individuals (females) that were sampled over the course of their development.

Following Czarnoleski et al. (2005) and Weinberg and Helser (1996), we used an approximate randomization method to examine the statistical significance of growth curve differences between the treatment groups. To compare two curves, we fitted Bertalanffy’s curves to two datasets (1 and 2), separately for each set, obtaining two curves with respective residual sums of squares, $SS_1$ and $SS_2$. After pooling the data from the two datasets, we fitted Bertalanffy’s curve to the pooled data (1+2), obtaining a new residual sum of squares $SS_{1+2}$. This method considers whether the two curves being compared are identical; the total residual sum of squares does not change after fitting a single curve to the pooled data ($SS_1 + SS_2 = SS_{12}$), but it increases if the curves have different shapes ($SS_1 + SS_2 < SS_{12}$). Therefore, the statistic $Diff = SS_{12} - (SS_1 + SS_2)$ measures the shape difference between two curves. If the curves are identical, then $Diff$ is equal to 0, but the more the curves differ, the higher the value of $Diff$ become. To assess the statistical significance of the shape difference in the curves, we compared the $Diff$ statistics with the distribution of their randomly generated values. The distribution was produced via 10,000 random shufflings of data. Each time, data from the two groups under comparison were pooled and randomly reassigned to the groups, keeping the number of observations per group the same as in the original groups. After each shuffling, we calculated $Diff^*$ according to the same procedure used to calculate $Diff$. Note that the distribution shows how frequently a given difference between curves ($Diff$) arises entirely from random processes, so when there is only
one general population, two groups of data on age and body size are drawn randomly. Statistical significance represented the probability ($p^*$ value) that the randomly generated $Diff^*$ statistic was as large or larger than the observed $Diff$ statistic. The randomization procedure was performed in R.

In our 2x2 experiment, thermal conditions (warm and cold) were crossed with oxygen conditions (normoxic and hypoxic). Consequently, to compare the growth curves between the warm and cold conditions, we had to simultaneously control the effects of oxygen conditions, and vice versa, i.e., the growth comparison between hypoxia and normoxia involved controlling the effects of thermal conditions. Therefore, to compare the curves between the warm and cold conditions, we calculated the $Diff$ statistic separately for the data obtained under normoxic and hypoxic conditions. After summing these two $Diff$s, we obtained a $Diff_{Temp}$ statistic, which measured the shape difference of the temperature curves while simultaneously integrating information from the normoxic and hypoxic conditions. Note that when the thermal conditions do not affect the growth curve, i.e., the curve is consistent between hypoxic and normoxic conditions, then $Diff$ calculated for normoxic conditions equals 0 and $Diff$ calculated for hypoxic conditions also equals 0, which leads to $Diff_{Temp}=0$. The more the growth curves differ between temperatures, and the more consistent this effect is in normoxic and hypoxic conditions, the higher the value of $Diff_{Temp}$. If the temperature and oxygen have interactive effects on the growth curves, e.g., when $Diff = 0$ for normoxic conditions (no thermal effects on growth curves in normoxic conditions) and $Diff > 0$ for hypoxic conditions (thermal effects on growth curves in hypoxic conditions), $Diff_{Temp}$ has an intermediate value. To compare the curves between normoxic and hypoxic conditions, we calculated the $Diff$ statistic separately with the data obtained from warm and cold conditions, and summing these values, we obtained $Diff_{Oxy}$. This statistic measured the shape differences in the oxygen level curves, integrating information obtained under either warm or cold conditions. Finally, with reference to our randomly generated distributions (see previous paragraph), we evaluated the statistical significance of $Diff_{Temp}$ and $Diff_{Oxy}$.

**Results**

*Proxy of lung size*

Validating our proxy of lung size, we found significant positive correlations between the area of the whole exopodites and the total area of their dark areas with pseudotracheae (developing woodlice: 0.92 (1st pair of exopodites) and 0.92 (2nd pair of exopodites), adult males: 0.86 (1st pair of exopodites) and 0.50 (2nd pair of exopodites), adult females: 0.60 (1st pair of exopodites) and 0.80 (2nd pair of exopodites)).
**Exopodite area**

When we analyzed the data on female woodlice in the course of their growth and development (Table 1; Fig. 3), we found a significant relationship between body mass and exopodite size ($p<0.001$). There was also a significant body mass and oxygen interaction ($p=0.01$). The temperature and log body mass interaction was close to significance ($p=0.07$), so we decided not to ignore it in our interpretation of model results. To improve the interpretability of our model with two interactions, we examined the results of the same model after centering the covariate (log body mass) at its two extreme and mean values (Fig. 3). Small animals had smaller exopodites in hypoxic conditions than in normoxic condition ($t=-2.04$, $p=0.04$); the effect of temperature was not significant ($t=1.22$, $p=0.22$).

In average-sized animals, we did not find effects of oxygen level on exopodite area ($t=0.92$, $p=0.36$), but increased temperature resulted in decreased exopodite area ($t=1.94$, $p=0.05$). Large animals had larger exopodites in hypoxic conditions than in normoxic conditions ($t=2.66$, $p=0.01$) and relatively smaller exopodites at increased temperatures ($t=2.63$, $p=0.01$).

In mature woodlice (Table 2; Fig. 4B), we found a significant increase in exopodite size associated with body mass ($p<0.001$). Interestingly, there was a significant sex and body mass interaction ($p=0.05$); in females, this relationship was more prominent (the scaling exponent equaled 0.64) than that in males (the scaling exponent equaled 0.57). Regardless of sex, increased temperatures reduced the mean area of exopodites ($p=0.004$, Fig. 4A). There was a significant oxygen and sex interaction ($p=0.002$), with exopodites being larger under hypoxic conditions than normoxic conditions, but only in males ($t=4.08$, $p<0.001$; Fig. 4B). In females, the size of the exopodites was not significantly different between oxygen groups ($t=1.36$, $p=0.41$, Fig. 4B). As the sex and covariate interaction was significant, we further analyzed the differences between the sexes, centering the covariate (log body mass) at three different values. Males had consistently larger exopodites than females at the minimal ($t=8.76$, $p<0.001$), mean ($t=16.81$, $p<0.001$) and maximal ($t=7.59$, $p<0.001$) log body mass, but this sexual dimorphism was less visible among large woodlice.

**Growth curves**

Fig. 5 shows the estimated Bertalanffy’s growth curves for growing females, indicating that the thermal treatment had a more pronounced effect on growth than did oxygen availability. We experienced computational problems in obtaining 95% confidence intervals for some values of growth curve parameters, which prevented us from comparing the growth curve parameters using a single, consistent significance level. Therefore, we calculated
confidence levels with the highest possible significance level (Table 3). Our randomization tests showed that the shape of the curves differed between the temperature treatments in both of the oxygen treatments ($p^*<0.0001$, Fig. S1). Cold-reared woodlice under either hypoxic or normoxic conditions were consistently characterized by an increased asymptotic body mass and a relatively lower value of the growth rate coefficient (Table 3). No significant effect of the oxygen treatment on the shape of Bertalanffy’s curve was detected ($p^*=0.87$, Fig. S1).

Discussion

Our study of *P. scaber* demonstrated that large woodlice developed relatively large pleopodite-exopodites with lungs, which was evident among both growing subadult woodlice and adults with different body sizes. In isopods, similar results were reported for the aquatic species *Asellus aquaticus* and *Saduria entomon*, demonstrating an allometric relationship between respiratory surface and body mass (Bielawski and Babula, 1980). This prevailing relationship likely indicates that an increased demand for oxygen due to an increased body size is fulfilled by an increased gas exchange surface. A positive relationship between body size and the size of the gas exchange organs was previously also found in turtles (Hochscheid et al., 2007), bats (Maina et al., 1991), fish, mammals, lizards and birds (Hughes, 1984). Consistent with the principle of symmorphosis, organisms are expected to coordinate development to match the physiological capacities of different tissue and organs (Taylor and Weibel, 1981), so a change in the size of an isopod’s lungs should be paralleled by adequate changes in other elements of the oxygen transportation system. However, in contrast with the expectations of symmorph, mammals have been shown to develop excessive gas exchange capacity in the lungs in relation to the capacities of other elements of the oxygen transportation system (Weibel et al., 1991). Certainly, a better understanding of the capacity of the gas exchange system in isopods would require information on cuticular gas exchange (Edney and Spencer, 1955), the amount and binding efficiency of hemocyanin, the volume and flow rates of hemolymph, and the diffusion of oxygen through the tissue and cells.

The results of our experiment demonstrated that the size of the gas exchange organs in woodlice developmentally responded to environmental conditions, which, to our knowledge, might be the first report of this kind of phenotypic plasticity in isopods. In line with the general idea that the gas exchange surface of isopod lungs is matched to the metabolic demand and oxygen supply, we found that the size of *P. scaber* lungs was sensitive to thermal and oxygen conditions during development. Nevertheless, the pattern of these responses suggests a compromise between the rates of oxygen uptake and water loss. It is
especially notable that the size of lungs increased differently during ontogenetic growth of *P. scaber* females, depending on the developmental temperature. Small woodlice had similar sized lungs irrespective of thermal conditions, but among large woodlice, warm woodlice had smaller lungs than cold woodlice (Fig. 3). Consistently, when we analyzed our dataset for mature males and females, we found that adult woodlice originating from our warm treatment had smaller lungs than the cold-reared individuals (Fig. 4). The development of relatively smaller lungs under warm conditions does not follow the expectation that a larger gas exchange surface helps ectotherms in warm conditions meet their increased demand for oxygen. However, the advantage of large lungs in warm environments can be reduced by the positive effect of temperature on oxygen diffusion rates combined with the increased rates of water evaporation via the gas exchange surface. This scenario agrees with the macroevolutionary trends of covering the lungs and limiting possible surface evaporation, which occurs according to the adaptation of species to more xeric environments (Hoese, 1982; Hsia et al., 2013; Schmidt and Wägele, 2001).

Consistent with the idea that an increased gas exchange surface facilitates oxygen uptake in hypoxic environments, we found that among mature adults, males reared under hypoxic conditions had larger lungs than those reared under normoxic conditions. The same response pattern also occurred in females, but only among growing individuals with a relatively larger size: as females were growing in size, their lungs were increasing faster under hypoxic conditions compared with normoxic conditions. Intriguingly, the opposite effect of oxygen conditions characterized growing subadult females at smaller sizes (but notably, younger and thus smaller females were exposed to the oxygen treatment over a shorter time interval than the larger and older females). In addition to the lungs, isopods also use the outer surfaces of other body parts for gas exchange with the environment (Edney and Spencer, 1955); this effect might explain why hypoxic conditions caused large woodlice, with a relatively low surface-to-volume ratio: large woodlice benefit more from large lungs than small woodlice. This effect, however, cannot explain why males and females responded differently to oxygen conditions in their lung size; among adults, the lungs of males became larger under hypoxic conditions than those of males under normoxic conditions, but the lungs of females remained the same size under different oxygen conditions. Notably, *P. scaber* males developed relatively larger lungs than females, so it appears that the sex with the relatively larger lungs (males) responded to hypoxia by enlarging the lungs, while the sex with the relatively smaller lungs did not respond to oxygen conditions. The sexual dimorphism of lung size and the sex differences in the oxygen dependence on the basis of lung size suggest that males might have higher metabolic demand for oxygen than females.
Indeed, resting *P. scaber* males tended to have higher rates of CO$_2$ production than females under controlled temperature and humidity conditions (Antol et al. unpublished results). Spiders have evolved intersexual metabolic differences, though which sex has an increased metabolic rate depends on the species (Kotiaho, 1998; Tanaka and Itô, 1982; Watson and Lighton, 1994). In contrast, mice and mosquitofish show little or no intersexual difference in metabolic rates (Cech et al., 1985; Schulz et al., 2002). Given the reproductive biology of isopods (Kight, 2008), increased metabolic demands might be expected in females rather than in males of *P. scaber* because females carry offspring in their marsupial pouches and provide offspring with resources and oxygen. Nevertheless, we consider that females might be more limited by the water supply because they supply their offspring with aqueous conditions for a considerable part of their marsupial development (Horváthová et al., 2017). In fact, Horváthová et al. (2017) demonstrated that females of *P. scaber* manipulate the duration of the aqueous phase inside the marsupium according to oxygen conditions in the environment. If so, we hypothesize that female terrestrial isopods might be under stronger selective pressure than males to compromise their capacity to obtain oxygen for the benefit of water conservation.

Our data on growth under thermal and oxygen treatments indicated that the oxygen conditions in our experiment did not significantly modify the growth rates, but cold-reared woodlice grew significantly slower and obtained larger asymptotic body mass than warm-reared woodlice. This result is consistent with our previous study on the growth of woodlice during their first 13 weeks of postmarsupial development, where we did not find an effect of oxygen (Horváthová et al., 2015a). A decrease in the asymptotic size of woodlice in response to warm rearing conditions is generally consistent with the predictions of the so-called temperature-size rule (TSR), a thermal plasticity pattern that has evolved in many ectotherms (Atkinson, 1994). Thus far, the thermal dependence of growth patterns has rarely been studied in isopods. On a geographic scale, the woodlouse *Porcellio laevis* follows the TSR and exhibits an increased body size in Chilean populations that are located further from the equator (Lardies and Bozinovic, 2006). Moreover, the TSR was confirmed experimentally in the freshwater isopod *Asellus aquaticus* under hypoxic conditions (Hoefnagel and Verberk, 2015). Our finding that oxygen conditions did not modify the growth curves of *P. scaber* does not follow the theory that the TSR is influenced by changes in oxygen availability combined with oxygen demand (Czarnoleski et al., 2015; Hoefnagel and Verberk, 2015; Walczyńska et al., 2015). Certainly, the nonsignificant effects of oxygen availability on the growth curves in our experiment might be caused by relatively good access to oxygen in our hypoxic treatment (10%), especially for litter dwelling animals.
such as *P. scaber*. It is also possible that the developmental plasticity of the lungs revealed in our experiment indicates compensatory changes that reduce the effect of oxygen availability on growth capacity. In nature, isopods are challenged with desiccation risk, which was minimized in our experiment, as we kept the animals under conditions with the highest possible humidity. The high humidity might have allowed them to develop lungs large enough to maintain an adequate oxygen supply.

Our previous study (Antol et al., 2019) demonstrated that the woodlouse *P. scaber* responded behaviorally to acute hypoxia by choosing cooler sites, which possibly helped survival by decreasing the oxygen demand. Here, we demonstrated that during development in different oxygen and thermal conditions, *P. scaber* underwent changes in the size of the gas exchange organs, which might be explained by a balance between the capacity to obtain oxygen and conserve water. Oxygen availability, thermal conditions and water conditions might be key environmental factors involved in the early evolution of terrestrial isopods (Horváthová et al., 2017). Approximately 300 Mya, after isopods invaded land, oxygen availability drastically dropped (by 50%; Berner et al., 2007), and this shift coincided with the evolutionary diversification of land isopods. During that time, this group of crustaceans evolved numerous adaptations (such as lungs), which must have played a role in the colonization of various terrestrial habitats. Our results suggest that this evolution preserved genotypes that have the capacity to respond in a plastic way to developmental conditions, which would help explain why terrestrial isopods thrive among a wide range of environmental gradients.

**Authors’ contributions**

- AML, MC and JK designed the study;
- AA and TH performed the experiment;
- AML and NSz performed the histological dissections;
- AML photographed the exopodites;
- AML and AP prepared and analyzed the (SEM) images;
- YV and BZ designed the image analysis software;
- AA collected and analyzed the data and drafted the manuscript;
- JK provided financial support for the study;
- AML, JK, MC and NSz critically contributed to the final stage of the manuscript;
- All authors have approved the final version.
Conflicts of interest

The authors declare no conflicting interests.

Acknowledgements

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Tables

**Table 1** Results of the general linear model comparing the exopodite areas (proxy of lung size) in female *Porcellio scaber* during their postmarsupial development at two oxygen levels (10% and 22%) and two temperatures (15 and 22°C).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log body mass</td>
<td>1</td>
<td>53.02</td>
<td>53.02</td>
<td>9340.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temperature</td>
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<td>0.02</td>
<td>0.02</td>
<td>3.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.7</td>
<td>0.41</td>
</tr>
<tr>
<td>Log body mass x temperature</td>
<td>1</td>
<td>0.02</td>
<td>0.02</td>
<td>3.4</td>
<td>0.07</td>
</tr>
<tr>
<td>Log body mass x oxygen</td>
<td>1</td>
<td>0.04</td>
<td>0.04</td>
<td>6.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Error</td>
<td>327</td>
<td>1.86</td>
<td>0.01</td>
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<td></td>
</tr>
</tbody>
</table>
Table 2 Results of the general linear model comparing the areas of exopodite (proxy of lung size) among mature adult *Porcellio scaber* males and females reared under different oxygen levels (10% and 22%) at two temperatures (15 and 22°C).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
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<tr>
<td>Log body mass</td>
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<td>3.279</td>
<td>3.279</td>
<td>1143.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temperature</td>
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<td>0.027</td>
<td>0.027</td>
<td>9.5</td>
<td>0.003</td>
</tr>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>0.013</td>
<td>0.013</td>
<td>4.7</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.702</td>
<td>0.702</td>
<td>244.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex x oxygen</td>
<td>1</td>
<td>0.033</td>
<td>0.033</td>
<td>11.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log body mass x sex</td>
<td>1</td>
<td>0.011</td>
<td>0.011</td>
<td>4.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Error</td>
<td>113</td>
<td>0.324</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Von Bertalanffy’s growth curve ($M_t = M_A (1 - e^{-K(t-t_0)})^3$) parameters for female *Porcellio scaber* that developed under two oxygen levels (10% and 22%) combined with two temperatures (15 and 22°C). $M_A$ – asymptotic body mass, $K$ – growth rate coefficient, $t_0$ – a hypothetical age at which zero size would be achieved. Confidence intervals (CIs) were estimated at the highest possible significance level. Graphical visualization of curves is shown in Fig. 5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia 22°C (75% CI)</th>
<th>Normoxia 15°C (75% CI)</th>
<th>Hypoxia 22°C (95% CI)</th>
<th>Hypoxia 15°C (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_A$ [mg]</td>
<td>48.48 (39.46–97.22)</td>
<td>68.42 (51.82–173.86)</td>
<td>44.44 (37.98–71.99)</td>
<td>56.96 (44.48–179.81)</td>
</tr>
<tr>
<td>$K$</td>
<td>0.002 (0.0006–0.004)</td>
<td>0.0006 (0.0002–0.001)</td>
<td>0.003 (0.0008–0.005)</td>
<td>0.0007 (0.0001–0.001)</td>
</tr>
<tr>
<td>$t_0$ [day]</td>
<td>120.6 (74.63–141.65)</td>
<td>136.3 (65.63–176.00)</td>
<td>127.6 (58.06–152.16)</td>
<td>123.5 (10.04–174.05)</td>
</tr>
</tbody>
</table>
Fig. 1 A structure of an exopodite with pleopodal lungs in *Porcellio scaber* (imaging with scanning electron microscopy). **A:** A female exopodite of the first pair of pleopods – a general view. **B:** Cells of the perispiracular area. **C:** Surface of the pleopodal lungs. Abbreviations: **cl,** connection of exopodite lamella with the base of pleopod; **lu,** lung branches; **mf,** microfolds of the external surface of the perispiracular cells; **pa,** cuticulized perispiracular area; **s,** funnel-shape area leading into a spiracle.
Fig. 2 Light microscopy images of pleopodal exopodites in females (upper panel) and males (lower panel) of Porcellio scaber (A, C – the first pair of pleopods and B, D – the second pair of pleopods). The photos were used to measure the area of flat-lying exopodites, our proxy of lung size. Abbreviations: cl, connection of exopodite lamella with the base of the pleopod; la, lung area; pa, cuticulized perispiracular area; s, funnel-shape area leading into a spiracle.
Fig. 3 The effect of oxygen or temperature on exopodite size in female *Porcellio scaber* undergoing postmarsupial growth and development. The means (± 95% CIs) were estimated with a general linear model with temperature or oxygen as fixed predictors after centering at three values of a covariate (log body mass). Significant differences are marked with different letters.
Fig. 4 In fully grown adult males and females of *Porcellio scaber*, exopodites were decreased with increased rearing temperatures (A), and their area increased with body mass (B). Males generally developed larger exopodites than females, but the size-dependence of exopodites was less in males than in females; males under hypoxic conditions had larger exopodites than males under normoxic conditions, but oxygen conditions did not affect the exopodites in females (B). The means (± 95% CIs) and regressions (females: hypoxia: $y=12.44+0.64x$, normoxia: $y=12.46+0.64x$; males: hypoxia: $y=12.95+0.57x$, normoxia: $y=12.91+0.57x$) were estimated with a general linear model with log body mass as a covariate and sex, oxygen and temperature as fixed predictors.
Fig. 5 Von Bertalanffy’s growth curves for female *Porcellio scaber* reared from eggs to adults under two oxygen levels (10% and 22%) combined with two temperatures (15 and 22°C). The curves for the actual range of data used in the curve fitting are displayed; Table 3 provides curve parameters.
Distribution of the randomly generated statistics $Diff_{Temp}^*$ (A) and $Diff_{Oxy}^*$ (A), which were used in an approximate randomization method to assess the statistical significance of differences in von Bertalanffy’s growth curves among the woodlouse *Porcellio scaber* from the experiment. Female woodlice were reared from eggs to adulthood at two temperatures (15 and 22°C) combined with two oxygen levels (10% and 22% O$_2$). To assess the effects of the treatments on growth, von Bertalanffy’s growth curves were fitted to data on the age and body mass of the woodlice, and the statistics $Diff_{Temp}$ and $Diff_{Oxy}$ were calculated to measure the differences in the growth curves between oxygen and temperature treatments, respectively. After that, the analyzed datasets were pooled and shuffled 10,000 times to establish the values of $Diff_{Temp}^*$ and $Diff_{Oxy}^*$ obtained via random re-assignment of data to experimental groups, $Diff_{Temp}^*$ and $Diff_{Oxy}^*$, respectively. The resulting distributions of $Diff_{Temp}^*$ (A) and $Diff_{Oxy}^*$ (B) show how frequently a given difference between the curves arises entirely from random processes, so when there is only one general population, the two groups of data on age and body size are drawn randomly. The statistical significance was the probability ($p^*$ value) that the randomly generated statistic $Diff_{Temp}^*$ (A) or $Diff_{Oxy}^*$ (B) was as large or larger than the observed statistic, $Diff_{Temp}$ (red line in (A)) and $Diff_{Oxy}$ (blue line in (B)).
STUDY III

Effects of thermal and oxygen conditions during development on cell size in the common rough woodlice *Porcellio scaber*

Submitted to Ecology & Evolution
Effects of thermal and oxygen conditions during development on cell size in the common rough woodlice *Porcellio scaber*

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Key words
life history evolution, metabolic rate, optimal cell size, temperature-size rule
Abstract
During development, cells may adjust their size to balance between the metabolic demand of tissue and the supply of oxygen and resources: a small cell might effectively absorb oxygen and nutrients, but its relatively large area of cell membranes requires costly maintenance. Consequently, warm and hypoxic environments should favour ectotherms with small cells to meet increased metabolic demand by oxygen supply. To test these predictions, we compared cell size (hindgut epithelium, hepatopancreas B cells, ommatidia) in common rough woodlice (Porcellio scaber) that were developed under four developmental conditions designated by two temperatures (15 or 22 °C) and two air O₂ concentrations (10% or 22%). To test whether small-cell woodlice cope better under metabolic demand, CO₂ production of each woodlouse was measured under cold, normoxic conditions and under warm, hypoxic conditions, and the magnitude of metabolic increase (MMI) was calculated. Cell sizes were highly intercorrelated, indicative of organism-wide mechanisms of cell cycle control. Cell size differences among woodlice were largely linked with body size changes (larger cells in larger woodlice), to a lesser degree with oxygen conditions (development of smaller cells under hypoxia), but not with thermal conditions. Developmental conditions did not affect MMI, and contrary to predictions, large woodlice with large cells showed higher MMI than small woodlice with small cells. We also found a complex pattern of sexual differences in the size of hepatopancreatic cells and the size and number of ommatidia, indicative of sex differences in reproductive biology. We conclude that existing theories about the adaptiveness of cell size do not satisfactorily explain the patterns in cell size and metabolic performance observed here in P. scaber. Thus, different organismal elements involved in sustaining the metabolic demands of tissue, including not only cell size but also characteristics of gas exchange organs, O₂-binding proteins, etc., should be simultaneously considered.

Introduction
Oxygen supply is usually considered a potential limiting factor for aquatic organisms (Bonvillain et al., 2015; Czarnoleski et al., 2015a; Hoefnagel and Verberk, 2015; Kielbasa et al., 2014; Verberk et al., 2016; Walczyńska et al., 2015; Woods, 1999). Nevertheless, obtaining adequate amounts of oxygen might also be challenging for terrestrial organisms, especially in habitats with high microbial respiration, periodic flooding or covered with snow, or simply if organisms live at high elevations (Hoback and Stanley, 2001; Paim and Beckel, 1964; Peacock, 1998). The need to supply oxygen to meet the metabolic demand of tissue has led to the selection of organisms with various adaptations, such as behavioural avoidance of warm microenvironments (Antól et al., 2019; Hicks and Wood, 1985; Wood and Gonzales,
1996), the ability to access alternative oxygen sources (e.g., air oxygen for aquatic organisms; Scott et al., 2017) or the ability to switch to anaerobic metabolism (Wright and Ting, 2006). Here, we consider that plastic cell size responses to environmental conditions might be another adaptation for satisfying metabolic oxygen needs. The adaptive value of cell size has long been overlooked despite accumulating evidence that it can co-vary with body mass within species (Arendt, 2006; Czarnoleski et al., 2013) and between species (Czarnoleski et al., 2018; Kozłowski et al., 2010), change with genome size (Gregory, 2001) and cell ploidy (Hermiuk et al., 2017, 2016), and respond to developmental conditions such as temperature (Arendt, 2006; Azevedo et al., 2002; Czarnoleski et al., 2015b; French et al., 1998; Hermiuk et al., 2016), oxygen supply (Heinrich et al., 2011; Zhou et al., 2007) and food supply (Arendt, 2006). Interestingly, although it appears that cell size changes are synchronized organism-wide, organs and tissue can group according to the size-responsiveness of their cells (Czarnoleski et al., 2018, 2017, 2015b; Kozłowski et al., 2010).

The theoretical framework for our work, namely, the theory of optimal cell size (TOCS), integrates ideas about the adaptiveness of cell size developed by earlier works (Atkinson et al., 2006; Czarnoleski et al., 2018, 2017, 2015b, 2013; Davison, 1956; Hermiuk et al., 2016; Kozłowski et al., 2003; Maciak et al., 2014; Szarski, 1983; Walczyńska et al., 2015; Woods, 1999). In line with Szarski (1983) and Kozłowski et al. (2003), TOCS considers that organismal strategies span a frugal-wasteful continuum largely dictated by cell size, with a frugal strategy (large cells in tissue) having low metabolic requirements at the costs of poor capacity to maintain high activity and rapid development compared with a wasteful strategy (small cells in tissue). In understanding the link between cell size and physiology, it should be considered that cell membranes are important transport hubs that affect the physiological capacity of cells; however, their operational state requires building and maintaining ionic gradients on the cell surface and turning over phospholipids, and such maintenance requires substantial amounts of ATP and resources (Engl and Attwell, 2014; Rolfe and Brown, 1997). Consequently, TOCS predicts that frugal strategies would connect with larger cells, which would reduce the amount of cell membrane per tissue/organ volume and, thus, the physiological load caused by cell membrane maintenance. In contrast, wasteful strategies would involve organs and tissue built of smaller cells. On the other hand, TOCS considers that organs built of smaller cells with more cell membranes might have an increased capacity to deliver oxygen through tissue to mitochondria. This is because oxygen diffuses better in lipids than in water, and thus, its diffusion through tissue should be facilitated inside phospholipid membranes compared with the aqueous cytosol inside cells (Subczyński et al., 1989). Ultimately, the theoretical framework of TOCS considers that
the fitness cost and benefit of a heavily wasteful vs frugal strategy depends on the selective pressure of the environment, especially the balance between metabolic demand and oxygen supply. In particular, wasteful strategies with smaller cells would be adaptive in warm and hypoxic conditions, whereas frugal strategies with large cells would benefit organisms in cold and normoxic conditions.

To address the predictions of TOCS, we studied the effects of thermal and oxygen conditions during development on cell size in the common rough woodlice (*Porcellio scaber*), a species of terrestrial isopods. Typically, terrestrial isopods inhabit litter environments with intense decomposition and thus are likely to experience hypoxic conditions in their natural habitats (Wright and Ting, 2006). Having evolved various land adaptations, such as pleopodal lungs, water conducting systems or conglobating behaviour (Cloudsley-Thompson, 1988; Hornung, 2011), terrestrial isopods are sometimes regarded as the best land adapted order of crustaceans (Hornung, 2011). Previous studies of terrestrial isopods showed that oxygen deficiency in the air affects their mobility, thermal performance and thermal preferences (Antol et al., 2019), respiration rate and haemolymph lactate level (Wright and Ting, 2006), lung size (Antol et al., under review) and the length of aquatic/air phases during marsupial development of offspring (Horváthová et al., 2017). Here, we assessed the cell size of woodlice in three different organs (eye, hepatopancreas and hindgut) involved in different organismal functions (vision, secretion and digestion). To our knowledge, no earlier study has focused on the cell size of isopods, especially considering the plastic developmental responses of cells to environmental conditions. Additionally, most earlier studies concerning cell size focused on single cell types, assuming that cells undergo coordinated growth and proliferation in different tissues (Heinrich et al., 2011; Starostová et al., 2013). Thus, our approach allowed us to not only assess the effects of ambient temperature and oxygen supply on cell size but also test the generality of these responses at an organismal level. Following TOCS, we predicted that the cell size of woodlice would decrease in response to either elevated metabolic demands (warm) or lowered oxygen supply (hypoxia), and we expected that this effect would be more pronounced under combined warm and hypoxic conditions (hypothesis 1). Before we sampled tissues for the determination of cell size, we assessed the capacity of each animal to meet increased metabolic demand under poor oxygen supply. For this purpose, we measured the magnitude of a metabolic increase, following a transfer of each woodlouse from cool and normoxic conditions to warm and hypoxic conditions. We predicted that small-cell woodlice would better tolerate such a challenge, which would be manifested by a higher increase in metabolic rate than large-cell conspecifics (hypothesis 2).
Material and methods

Animals

This work was part of a long-term study of *P. scaber*, and its methods are already available elsewhere (e.g., Horváthová et al., 2015a). Briefly, adults of *P. scaber* were collected in autumn (2013) and spring (2014) in an old backyard in Kraków, Poland (50°04'15.9"N 19°56'21.9"E). After sexing, females and males were transferred to separate plastic boxes with moist sand and broken clay pots as shelters, and the boxes housing the animals were kept in a thermal cabinet (Pol-Eko Aparatura, Wodzisław Śląski, Poland) set to 15 °C during the day and to 8 °C at night with 12L:12D photoperiod. Note that these conditions mimicked autumn/spring conditions in the source population. Animals were fed *ad libitum* with a mixture of dry black alder (*Alnus glutinosa*) and European ash (*Fraxinus excelsior*) leaves from a nearby forest. Note that the field-collected woodlice served as the parental generation for the new lab-created generation of woodlice, which was subjected to our developmental experiment.

Developmental experiment

The experiment aimed to allow woodlice to develop in one of four environments corresponding to each combination of two temperatures (15 °C and 22 °C) and two oxygen concentrations in the air (10% and 22%). The environments were generated in two thermal cabinets (Pol-Eko Aparatura) set to either 15 °C or 22 °C, with the help of four plexiglass chambers (40 × 50 × 55 cm), with two chambers placed in each cabinet. One of the two chambers per cabinet was set to normoxia (22%), and the other was set to hypoxia (10%). The oxygen conditions inside the chambers were controlled by a four-channel gas regulator ROXY-4 (Sable Systems International (SSI), Las Vegas, NV, USA). The regulator was connected to nitrogen and oxygen gas tanks (Air Products Sp. z o.o., Kraków, Poland) to supply the normoxic chambers with oxygen or the hypoxic chambers with nitrogen in the amounts necessary to create the experimental oxygen levels. Inside the chambers, air circulation was maintained by fans, and the relative humidity was controlled by four dew point generators DG-4 (SSI) set to 75% relative humidity and connected to the chambers via PP2 pumps (SSI). The temperature and humidity inside the chambers were monitored with Hygrochron iButtons (Maxim/Dallas Semiconductor, San Jose, CA, USA) placed within the food leaves and shelters of the woodlice. Note that the relative humidity experienced by the experimental animals reached 98%, which closely resembles the microhabitat humidity of our source population (Horváthová et al., 2015a).
To obtain a new generation for the experiment, the field-collected parental woodlice were randomly allocated for mating in 28 boxes, with 40 males and 50 females per box. The mating groups were randomly allocated to one of the four experimental environments to ensure that all animals from the new generation had already been exposed to the treatment conditions at the egg stage. According to McQueen and Steel (1980), the mating of parental animals was synchronized and stimulated by a sudden switch to a long-day photoperiod (16L:8D). The mating groups were checked weekly for gravid females (eggs or juveniles in a brood pouch - marsupium). Gravid females were immediately placed in isolation in 100 ml plastic boxes (the habitat inside the boxes was created as described in the Animals section). Every week, the boxes with gravid females were sprayed with water, and leaves were added. If free living stages of offspring were observed, the female parent was removed from the box. The offspring were fed dry leaves every week (see Animals section). To ensure access to gut microsymbionts, two-week-old juveniles were provided with a body of a dead adult conspecifics (Horváthová et al., 2015b). From the 4th week of postmarsupial life, the offspring were supplemented with one dried mealworm per box per week (Tenebrio molitor). At the age of 20 weeks, we pooled experimental animals of the same sex from all boxes and formed single-sex groups (20 animals per group). Each group was placed in a box where the animals remained until the end of the experiment (adulthood). When the females reached a body mass indicative of mature woodlice in our source population in the field (76.83 mg on average, Antol and Czarnoleski, 2018), we added several males to each box with the females to provide conditions for physiological maturation (Kight, 2008). When the animals reached the age of 18 months at 22 °C or 28 months at 15 °C, we assumed their maturity and terminated the experiment. For the animals that survived to this stage, we conducted measurements of respiration rate under two conditions and then performed dissections for cell size measurements.

Initially, all four experimental treatments involved a new generation of woodlice produced by the parental animals collected in the field in autumn 2013. Unfortunately, due to failure of one of our thermal cabinets, we lost animals from the warm treatment. Given the expected developmental lag between cold and warm woodlice, we decided to continue the cold treatment and run the warm treatment again a few months later using descendants of the parental animals collected in spring 2014. Both samples of parental animals (autumn 2013 and spring 2014) originated from the same source population and were acclimated to the same laboratory conditions prior to egg laying, including mating stimulation via the transition through the winter/spring phases. Therefore, we have good reason to believe that the parental animals collected in the autumn and the spring gave birth to phenotypically
similar offspring, not biasing our comparisons of animals developed in the laboratory in cold and warm treatments.

**Magnitude of metabolic increase (MMI)**

Respiration measurements were carried out on adult woodlice available at the end of our experiment, and they aimed to test whether animals from different treatments (and, possibly, with different cell sizes) cope differently with metabolically challenging conditions. For this purpose, we measured the respiration rate of each animal twice: first in less demanding cold/normoxia conditions (15 °C combined with 22% O₂) and then in more demanding warm/hypoxia conditions (22 °C combined with 10% O₂). Ultimately, each animal was characterized by the magnitude of metabolic rate increase (MMI), calculated as the ratio of the latter to former respiration rate. Before and after respiration measurements, animals were maintained in their original experimental treatments, and they were kept in 100 ml plastic boxes with a leaf of European ash and black alder as food sources, a piece of wet paper as a water source and a piece of a broken clay pot as a shelter. Prior to respirometry measurements in either cold/normoxia or warm/hypoxia, animals were acclimated to each condition for two days.

For respiration measurements, we used an eight-channel MUX multiplexer (SSI) with 7 channels connected to respiratory chambers dedicated for animals and one empty channel for a baseline. We used glass tubes (length: 7 cm, diameter: 2 cm) as respiratory chambers. To limit the locomotor activity of animals, we decreased the volume of each respiratory chamber by placing a smaller plastic tube filled with artificial cotton wool inside the chambers. The respiration chambers were placed in a thermal cabinet for temperature control during the measurements. We obtained the respiration rate by measuring CO₂ production in a flow-through system (SSI). We used the gas stream either from the outside (in case of normoxia measurements) or from the tank with a 10% O₂:80% N₂ gas mixture (Gaz Centrum, Kraków, Poland) for measurements in hypoxia. Before entering the system, the inflowing gas was scrubbed for H₂O with the use of calcium sulphate (Drierte Co. Ltd, Xenia, USA) and for CO₂ with the use of soda lime (Drierte Co. Ltd). To pump the gases, we used an SS4 Subsampler (SSI). The flow rate was set to 40 ml/min with a mass flow controller (Sidetrack Mass Flow Controller, Sierra Instruments, Monterey, CA, OH, USA). The relative humidity inside the respiration chambers was set to 85% (regulated with a DG-4 Dewpoint Generator, SSI, and controlled with an RH-300, SSI). The air leaving the experimental chamber was dried with magnesium perchlorate (Merck KGaA, Darmstadt, Germany). Every second, an infrared CO₂ gas analyser (Li-7000, Li-Cor, Lincoln, NE, USA).
recorded the $\dot{V}_{CO_2}$ (in ppm) in the air leaving the experimental chamber and dried with magnesium perchlorate. For each animal, respiration data were recorded for 10 consecutive minutes, after which data from the baseline were recorded for 5 minutes. The recorded $\dot{V}_{CO_2}$ was converted to ml CO$_2$ min$^{-1}$, baseline- and drift-corrected with ExpeData software (SSI). Ultimately, for each animal, we calculated the mean CO$_2$ consumption during a 2.5-minute time interval when the mean rate of respiration reached its lowest value, first under cold/normoxia and then under warm/hypoxia, and these measurements were used to calculate MMI.

Cell size

After respiration measures, the animals were weighed to the nearest 0.001 mg on a microbalance and dissected to obtain the head, the hepatopancreas and the hindgut for cell size measures. Animals were decapitated with a scalpel in a Petri dish. The remaining body was submerged in 1x PBS (Avantor Performance Materials, Gliwice, Poland), and the hindgut and hepatopancreas were extracted from the body. Food residuals were washed out from the hindgut with 1x PBS, and afterwards, both organs were fixed in 10% buffered formalin (Chempur, Piekary Śląskie, Poland).

Each freshly cut head was used to photograph ommatidia in the eyes. In isopods, each ommatidium is formed by a constant number of ten cells (Nemanic, 1975), which allowed us to treat the size of ommatidium facets as a proxy of cell size. Additionally, the total number of ommatidia in the eyes was counted to explore whether changes in the size of ommatidia correspond to changes in the number of ommatidia in the compound eye. We photographed ommatidia in both eyes under 63x magnification with a uEye digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) and a stereoscopic microscope SZY 10 (Olympus, Tokyo, Japan). The heads were impaled on a pin mounted in plasticine and lit with ring light (KL-RL-9/1000-3, Olympus). First, we took overview photographs of the entire eye and the frontal and back parts of the eye. Then, the head was positioned to obtain a perpendicular orientation of the singular ommatidium to the camera. The perpendicularity was controlled with the central settlement of the position of the light reflected by the ommatidium facet (Fig. 1 A). Only ommatidia that were not along the border of the eye were photographed to avoid possible shape irregularities caused by contact with the head carapax. The ommatidia measurements were performed in ImageJ software (NIH, Bethesda, USA). We split each photograph into separate colour channels and performed measurements on the green channel to ensure the best visibility of ommatidia borders. After contrast
enhancement, we fitted an ellipse to each ommatidium and measured its area to the nearest 0.001 μm².

The fixed hepatopancreas was washed three times for 30 minutes in 1x PBS. Next, the organ was dehydrated in a graded series of ethanol (70, 80, 90, 96, 99.8%), cleared in ST Ultra (Leica, Wetzlar, Germany) and embedded in Paraplast Plus (Leica). Serial cross-sections (4-μm thick) were cut with a rotary microtome (Hyrax M55, Zeiss, Oberkochen, Germany). Histological slides were stained with Ehrlich haematoxylin (Carl Roth, Karlsruhe, Germany) for ten minutes and with a 1% ethanol solution of eosin Y (Analab, Warszawa, Poland) for five minutes. Then, slides were dehydrated in 96% ethanol and a mixture of phenol (Avantor Performance Materials) and xylene (Avantor Performance Materials), cleared in xylene (Avantor Performance Materials) and embedded in DPX (Aqua-Med Zpam-Kolasa sp.j., Łódź, Poland). We took photos of hepatopancreas cross-sections in ZEN image acquisition software (Zeiss) under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan) in a bright field using 200x microscope magnification. Using ImageJ software, we measured B cells (Hames and Hopkin, 1991) with visible nuclei and nucleoli (Fig. 1 B). We outlined cells along the cell membrane to measure the cell area to the nearest 0.001 μm². Then, we calculated the mean cell size per individual, and this value was used in the analysis.

The hindgut after fixation was washed two times for five minutes in 1x PBS and then stained in an Eppendorf-like tube for 30 minutes with Gill II haematoxylin (Carl Roth). Organs were washed in tap water, cut and placed in one drop of tap water on the surface of a glass slide and covered such that the internal hindgut epithelium was oriented towards the cover slide. We captured photographs of the epithelium under a light microscope (Eclipse 80i) in a dark field using 100x microscope magnification (Fig. 1 C) and measured the epithelial cell size according to the method proposed by Czarnoleski et al. (2017) for hepatocytes using ImageJ software. In each photograph, we outlined a circular area covering the largest area of the tissue with cells with visible nuclei. To calculate the mean size of the hindgut epithelial cells for each individual, we measured the area of all circles from that individual and divided the area by the total number of cells inside. Cells located on the border were included if the majority of their surface was inside the circle. If such classification was ambiguous, the cell was randomly classified at a rate of 50% as being located inside the area. Then, we counted the total number of cells in all circles per individual, and the mean cell area measured to the nearest 0.001 μm² per individual by dividing the total area of measured circles per individual by the total number of cells for that individual.

In total, we measured 4–22 ommatidia (average 14.2), 3–95 hepatopancreas cells (average 35.7), and 46–273.5 (average 136.4) hindgut epithelium cells per individual.
woodlouse. Note that these ranges mainly reflect a change in the number of cells available for measurements with woodlouse body size.

**Statistical analysis**

We collected a full dataset from 111 animals (61 males and 50 females), which included information on sex, body mass, magnitude of MMI and cell size measurements for each of the three cell types. Statistical analysis and graphic presentation of the results were conducted in R software (R Core Team 2018) with lme4 (Bates et al., 2015), lmerTest (Kuznetsova, 2017), effects (Fox and Weisberg, 2018) and ggplot2 (Wickham, 2016) packages. Prior to the analysis, data on cell size were normalized and then analysed with principal component analysis (PCA). This analysis was performed to (i) integrate information about cell size in different organs and (ii) evaluate cell size correlations among different tissues. In the subsequent analyses, we used scores of extracted principal components (PC) as indices of integrated information on cell sizes. Following Quinn and Keough, (2002), we considered that the variance explained by PCs with eigenvalues > 1 should have much higher biological relevance than the variance explained by PCs with eigenvalues < 1. Consequently, testing our a priori hypotheses (1 & 2), we focused primarily on PCs with eigenvalues >1.

To test hypothesis 1 (metabolic demand and oxygen supply during development induce developmental changes in cell size) and hypothesis 2 (higher MMI in woodlice with smaller cells), we analysed our cell size measures (PC scores) and MMI (as dependent variables) with the help of two separate general linear models (GLMs) that had the same structure: rearing temperature and oxygen conditions as two fixed factors, sex as another fixed factor, and body mass of woodlice as a numeric covariate. The models also included an oxygen x temperature interaction, which allowed us to explore whether the effects of increased metabolic demand (warm) are magnified by low oxygen supply (hypoxia). The MMI data were log-transformed prior to the analysis to meet the assumption of normality. Another GLM with a similar structure was used to analyse ommatidia number. If any of our GLM models revealed a significant interaction, we further explored differences between groups with Tukey’s HSD test.

**Results**

Our measures of cell size in different organs spanned $3.9 \times 10^3 - 7.6 \times 10^3$ (mean=$5.8 \times 10^3$) $\mu m^2$ for ommatidia, $4.5 \times 10^2 - 3.6 \times 10^3$ (mean=$18. x 10^3$) $\mu m^2$ for the hepatopancreas and $1.8 \times 10^3 - 7.2 \times 10^3$ (mean=$4.4 \times 10^3$) $\mu m^2$ for the hindgut epithelium. Following our PCA of the cell size data (Table 1), we considered two principal components, PC1 and PC2,
though only PC1 had a large enough eigenvalue (1.86) to regard it as significant (Quinn and Keough 2002). Note that although we further analysed the results of both PCs, our hypothesis testing involved the results of PC1, whereas the results of PC2 were analysed for explorative purposes.

The results of PC1 showed that cell size in the hepatopancreas and the hindgut and ommatidia size were positively loaded on PC1; therefore, higher scores indicate larger cells in these three cell types. The results of PC2 showed that the size of hepatopancreas cells and ommatidia were also partially loaded on PC2, but in opposite directions: higher scores were associated with larger hepatopancreatic cells and smaller ommatidia. The hindgut epithelium cell size did not contribute significantly to PC2. Note that the simultaneous contributions of cells in the hepatopancreas and ommatidia to PC1 and PC2 indicate that the variance in the size of these three cell types can be divided into two independent parts (though these parts are unequal because PC1 explained a much larger part of the variance than PC2), which have two different natures reflected in the structure of the two PCs.

The results of the GLM for PC1 scores (Table 2) showed that the scores increased with woodlouse body mass (p<0.001; Fig. 2). This means that larger woodlice were characterized by larger cells in ommatidia, hepatopancreas and hindgut epithelium. There was a tendency of PC1 scores to have lower values in hypoxia than in normoxia (p=0.07), indicating a trend towards smaller cells in ommatidia, hepatopancreas and hindgut epithelium in hypoxic woodlice than in normoxic woodlice. The effects of sex, temperature and the oxygen x temperature interaction were not significant.

The results of GLM for PC2 scores (Table 2) showed that males had smaller scores than females (p=0.006, Fig. 3B). This means that compared with females, males were characterized by smaller hepatopancreatic cells and simultaneously larger ommatidia. Temperature and oxygen imposed an interactive effect on PC2 scores (p=0.004). The highest PC2 scores were achieved by animals that underwent development in either 22 °C combined with 10% O₂ or in 15 °C combined with 22% O₂. These conditions resulted in small ommatidia and large cells in the hepatopancreas (Fig. 3A). Following our pairwise post hoc comparison of groups (Tukey’s HSD test), the difference closest to the significance threshold was observed between animals reared at 22 °C combined with 22% O₂ and animals reared at 15 °C combined with 22% O₂ (p=0.058). The body mass effect was insignificant (p=0.13).

The results of the GLM for the MMI (Table 2, Fig. 4) showed that upon transition from cold, normoxic conditions to warm, hypoxic conditions, MMI did not differ between sexes and did not depend on the rearing conditions of the woodlice. However, the model showed a significant effect of woodlouse body mass on MMI (p=0.03), indicating that
a transition to more metabolically demanding conditions imposed a stronger increase in metabolic rate among large vs small woodlice.

The results of the GLM for ommatidia number (Table 2, Fig. 5) showed that the number of ommatidia in the eye increased with woodlouse body mass (p=0.01), animals reared in warm conditions had more ommatidia than animals reared in cold conditions (p<0.001), females tended to have more ommatidia than males (p=0.09), and animals reared in hypoxia tended to have more ommatidia than animals reared in normoxia (p=0.06). The effect of the oxygen x temperature interaction was not significant (p=0.57).

**Discussion**

*Coordinated cell growth in tissues*

By integrating information on cell size in different organs of *P. scaber* (eye, hepatopancreas and hindgut) with our PCA, we found that the majority of cell size differences among individuals arose in a coordinated manner throughout the entire body, rather than occurring only in individual organs (see our PC1, Table 1, Fig. 2). The interlink between cell sizes in different organs/tissues has rarely been studied, but available evidence has revealed this phenomenon in different species, such as *Cornu aspersum* snails (Czarnoleski et al., 2015b) and *Paroedura picta* geckos (Czarnoleski et al., 2017). Furthermore, interspecific comparisons of Hawaiian flies (Stevenson et al., 1995), amphibians (Kozłowski et al., 2010), birds (Czarnoleski et al., 2018; Kozłowski et al., 2010), mammals (Czarnoleski et al., 2018) and plants (Brodribb et al., 2018; Kozłowski et al., 2010), mammals (Czarnoleski et al., 2018) and plants (Brodribb et al., 2013) as well as the results of experimental evolution in mice (Maciak et al., 2014) suggest that the cellular architecture of different organs can undergo highly coordinated evolution. Altogether, this emerging evidence highlights the importance of understanding the mechanisms of cell size control and organism-wide coordination. These two phenomena are still not well recognized, but accumulating evidence has indicated that they might operate via evolutionarily conserved cellular signalling that targets biochemical pathways involved in cell size control, such as the target of rapamycin (TOR) and insulin regulatory pathway (De Virgilio and Loewith, 2006; Grewal, 2009), or via alterations in genome size (Gregory, 2001).

*Cell growth contributes to an increase in body size*

As the largest portion of the variance in cell size was attributed to PC1, cell size differences among individual woodlice could be largely attributed to differences in body size and to some extent to oxygen conditions during development. These results show that organs of large woodlice consisted of larger cells than organs of small woodlice, which resembles
patterns revealed previously, e.g., in tadpoles of Spea hammondi toad (Arendt, 2006), D. melanogaster flies (Czarnoleski et al., 2013) or Lecane inermis rotifers (Walczyńska et al., 2015). Interestingly, covariance between cell size and body size was also observed at the interspecific level, which strongly suggests that body size and cell size can undergo concerted evolution during species divergence (Czarnoleski et al., 2018; Kozłowski et al., 2010). Certainly, a change in body size can occur not only via cell size changes but also via changes in cell number, which was previously demonstrated in western spadefoot toads (Arendt, 2006) and fruit flies (De Moed et al., 1997). In Drosophila flies, for example, alterations in cell number accounted for 66% of the variance in body size (Böhni et al., 1999; Minelli et al., 2010). For P. scaber studied here, we found that the number of ommatidia (eye elements built in isopods from a constant number of cells) increased in the eyes of larger woodlice, indicating that changes in cell number together with cell size contributed to changes in the size of eyes and possibly also in the body size of woodlice. Changes in cell size and cell number can also be viewed as components of ecological patterns in body size, which was envisioned to reflect adaptive shifts in life history, combined with tuning of cellular architecture to metabolic demands and supply of oxygen and resources (e.g., Atkinson et al., 2006; Czarnoleski et al., 2015a; Kozłowski et al., 2010). For example, ectotherms often show phenotypic plasticity that results in larger body size under colder rearing conditions (the so-called temperature size rule; Atkinson, 1994) or geographic clines that form by evolving larger sizes in colder climates and smaller sizes in warmer climates (the so-called Bergmann’s rule; Bergmann, 1848); evidence shows that these patterns are often associated with changes in cell size (Adrian et al., 2016; Partridge et al., 1994; van Voorhies, 1996; Zwaan et al., 2000). Interestingly, latitudinal clines in the body size of isopods that follow Bergmann’s rule have been reported in Porcellio laevis woodlice from Chile (Lardies and Bozinovic, 2006), a close relative of P. scaber, and it would be interesting to evaluate the contributions of cell size and cell number in these clines.

**Oxygen, temperature, cell size and metabolic performance**

In comparing our results with TOCS, we found a partial agreement with predictions that warm and oxygen-deficient conditions favour small cells (hypothesis 1). As expected, woodlice exposed to chronic oxygen deficiency during development tended (p=0.07) to have smaller cells consistently in all three studied tissue types (see PC1, Fig. 2). A link between the environmental supply of oxygen and cell size has been very rarely studied, but a decrease in cell size in response to environmental hypoxia was observed, e.g., in rotifers (Czarnoleski et al., 2015a; Walczyńska et al., 2015) and fruit flies (Heinrich et al., 2011; Zhou et al., 2007),
but not in bryozoans (Atkinson et al., 2006). Against TOCS predictions, thermal environment did not affect cell size in the studied woodlice (see PC1, Table 2, Fig. 2). We cannot tell whether this finding generalizes to other isopods because to our knowledge, no earlier works have addressed the effects of the thermal environment on isopod cell size. Nevertheless, our findings for *P. scaber* do not follow the results of many previous studies on other invertebrates, which demonstrated that animals originating from warmer or thermally variable environments were characterized by small cells (Adrian et al., 2016; Atkinson et al., 2006; Czarnoleski et al., 2015b, but see Arendt, 2006).

Given the framework of TOCS, we predicted that upon transitioning from cold, normoxic to warm, hypoxic conditions, metabolic performance would be less prone to oxygen limitation in small-cell vs large-cell woodlice (hypothesis 2). Our results only partially supported this hypothesis. As outlined above, rearing in different thermal environments did not change the cell size of woodlice, and consistently, the transition of woodlice to metabolically more demanding conditions did not cause different metabolic responses in woodlice originating from different thermal environments. However, against predictions, metabolic performance was comparable in woodlice originating from different oxygen conditions, regardless of cell size differences between hypoxic and normoxic woodlice (see Fig. 3, 4 and the previous paragraph). Surprisingly, our results showed that the transition from cold, normoxic to warm, hypoxic conditions resulted in a more pronounced increase in metabolic rate in large woodlice than in small woodlice, despite the large woodlice being composed of larger cells than small woodlice. We can only speculate on some possible explanations of this discrepancy in our predictions regarding the effects of cell size on metabolic performance, especially given that our data do not allow us to separate the effect of cell size from the effect of body size, as the two traits were strongly interrelated. For example, the properties of the gas-exchange system of woodlice (e.g., the lungs) might make larger individuals less prone to oxygen limitation than smaller individuals. We should also consider here that cell size and, thus, the total area of cell membranes in tissue might not affect metabolic performance if animals are studied in their resting state, as was the case here. In contrast to our findings, some previous studies have demonstrated an inverse relationship between cell size and metabolic rate (e.g., Chown et al., 2007; Czarnoleski et al., 2018; Hermaniuk et al., 2017; Maciak et al., 2014; Starostová et al., 2013, 2009). Also against the pattern revealed here for *P. scaber*, comparisons of thermal sensitivity of Carabidae beetles showed that large species were characterized by higher metabolic responses to thermal change than small species (Gudowska et al., 2017). Note, however, that these studies of metabolic rates focused primarily on differences in maintenance costs driven by cell size...
rather than on the capacity of cells to deliver adequate levels of oxygen under temporal fluctuations in metabolic demand and oxygen supply. In fact, there are arguments against extrapolating physiological phenomena observed in organisms during resting states to ecologically and evolutionarily relevant situations because resting metabolism can be a by-product of evolution of other characters (Clarke and Pörtner, 2010, Kozlowski et al. under review).

Residual cell size variance and ommatidia number

By focusing on a relatively small part of the cell size differences among woodlice captured by PC2 in this study (Fig. 3, Table 2), we found a very complex pattern: the size of hepatopancreatic cells was negatively related to the size of ommatidia, and these two types of cells changed their size independently of the size of epithelial cells in the hindgut. This pattern resembles earlier reports that cells in anabolically active organs involved in service functions, such as the liver in vertebrates (Czarnoleski et al., 2018, 2017) or the hepatopancreas in invertebrates (Czarnoleski et al., 2015b), can undergo changes in the opposite direction of other cell types. Consequently, our analysis of PC2 with reference to predictions that warm hypoxic conditions favour smaller cells (hypothesis 1) leads to inconclusive results. While the size of cells in two tissues (ommatidia and hepatopancreas) changed according to oxygen and thermal conditions during development, the size of cells in the hindgut epithelium was largely irresponsive to developmental conditions. Furthermore, when the size of one cell type decreased in response to high temperature, as predicted by hypothesis 1, the other cell type increased in size, contrary to hypothesis 1. However, this effect of temperature further depended on rearing oxygen level: in hypoxia, there were no thermal differences among cells, but in normoxia, cold temperature caused shrinkage of ommatidia and enlargement of hepatopancreatic cells. Despite the complexity of this pattern, the results of PC2 to provide some insights into potential sex differences in cell size. We found that females were characterized by higher PC2 scores than males, suggesting larger cells in the female hepatopancreas and smaller ommatidia in the female compound eye. According to Kubrakiewicz (1994) and Tseng et al. (2001), the hepatopancreas of crustacean females is involved in vitellogenin synthesis, but the hepatopancreas of isopod females serves only as the storage site for vitellogenin synthesized in fat bodies (Picaud, 1980; Vafopoulou and Steel, 1995). It is tempting to consider this characteristic of isopods in understanding sex differences in hepatopancreatic cells of P. scaber: larger hepatopancreatic cells in females may reflect the amount of vitellogenin storage, which is required by yolk production for oocytes. Note that our results of PC2 combined with the data on the number of ommatidia
indicate that *P. scaber* males were characterized by larger but fewer ommatidia than females (Fig. 5). Compared with *Ligia oceanica* isopods, in which the number of ommatidia reaches 700 (Edwards, 1969), or with insects that have in excess of 1000 ommatidia (e.g., *Asterocampa leilia* butterflies, Ziemba and Rutowski, 2000), we found that the eyes of *P. scaber* consisted of very few (20 – 30) ommatidia (Fig. 1 and 5). Therefore, it seems likely that one or two new ommatidia added to a small eye of *P. scaber* females might have an incomparably large effect on their visual capacity compared with eyes that contain much larger numbers of ommatidia. It is likely that improved vision might benefit more females than males: at gravidity, females suffer from increased exposure to predators because of their handicapped mobility (Kight and Ozga, 2001), and improved vision might help reduce predation risk. Sexual dimorphism in ommatidia number was already reported by earlier studies, but with different patterns in different species. Similar to our results in *P. scaber*, Ziemba and Rutowski (2000) found more and smaller ommatidia in female *A. leilia* butterflies, suggesting that this pattern relates to a mating strategy in this species: males do not search actively for mates but wait until a female appears in their close view. Opposite sex differences in eye composition were also reported for *Asaphidion sterlini* carabid beetles (Bauer et al., 1998), possibly indicating the active search for females by males. Interestingly, no intersexual differences in ommatidia size were reported in the solitary mason bees, *Osmia rufa* (Kierat et al., 2016), although males had larger ommatidia than females if both sexes were compared at equal body sizes.

Exploring further differences in the number of ommatidia among our experimental treatments, we observed more numerous ommatidia in animals reared at high temperature and a trend towards an increased number of ommatidia under hypoxia. Given that ommatidia size was not affected by temperature, changes in ommatidia number should be tightly linked to changes in the total size of the eye. Earlier works provided evidence suggesting that the eyes of crustaceans and insects might consist of ommatidia with fixed size, and thus, changes in eye size could be obtained exclusively by the addition of new ommatidia (Minelli et al., 2010). Nevertheless, ommatidia size was shown to vary according to rearing temperature in *D. melanogaster* (Azvedo et al., 2002) and *Osmia rufa* (Kierat et al., 2016) and between animals from different nests in *Formica rufa* ants (Perl and Niven, 2016a); it was also shown to change among regions of the eye, as demonstrated in *Asterocampa leilia* butterflies (Ziemba and Rutowski, 2000) and *F. rufa* ants (Perl and Niven, 2016b). Note that our results for *P. scaber* (PC1) also revealed changes in the size of ommatidia with body size and, to a lesser degree, with oxygen level in the environment (Table 2). Interestingly, we have good reason to expect that the size of ommatidia can vary according to the region of the eye.
in woodlice. While photographing, measuring and counting ommatidia in *P. scaber*, we observed that the smallest and most “packed” ommatidia were present in the frontal part of the eye (unfortunately, we did not measure these ommatidia for technical reasons). We suppose that upon consecutive moulting events, new ommatidia are added to the frontal edge of the eye. This phenomenon was already observed in scanning electron microscopy (SEM) photos of mancae developmental stages in closely related isopod species, such as *Porcellio dilatatus* (Brum and Araujo, 2007; Figs: 46, 47) and *Ligia exotica* (in crescentic dorso-anterio-ventral edge of eye) (Keskinen et al., 2002).

**Conclusions**

Overall, we conclude that factors shaping cell size and metabolic performance in *P. scaber* appear more complex than predicted by TOCS and cannot be fully attributed to the effects of thermal and oxygen conditions in the environment. We observed a rather weak influence of rearing conditions on cell size: a tendency towards smaller cells under hypoxia was revealed in the major component of cell size differences among woodlice (our PC1, Fig. 2) and a very complex cell size pattern was revealed by the smaller component of cell size differences among woodlice (our PC2, Fig. 3). We also found strong evidence that cell size changes (PC1) represent the main mechanistic driver of body mass increase in *P. scaber*. It would be interesting to further explore this phenomenon, for example, by checking whether cell size changes are linked to a notable tendency of this species to continue growth after maturation (Antol and Czarnoleski, 2018), as it has been reported that the growth of isopods is sustained from the “capital” of energy and chemical elements assimilated between moults, occurring over a very restricted time interval after shedding the external exoskeleton (Warburg, 1993). We also revealed two intriguing patterns: a sexual dimorphism of the eyes, with female woodlice having more ommatidia than male woodlice, and an increased metabolic capacity of larger woodlice that consisted of larger cells in a body. We envision that a better understanding of these sex differences would require the consideration that female eyes might be adapted to counteract an increased risk of predation during brooding. To better understand the links among body size, cell size and metabolic performance, future studies should evaluate metabolic performance at different activity levels and consider the transport efficiencies of different elements of the oxygen delivery system.
Author contributions
AML, MC, JK designed the study;
AA, AML, MC, JK and UB designed the methodologies;
AA and TH performed the experiments and respiration rate measurements;
AML, AS, NS performed the dissections;
AML and AS prepared the histological slides;
AA collected the data;
AA and MC analysed the results and
AA led the writing of the manuscript.
All authors critically contributed to the manuscript and gave final approval for publication.

Conflicts of interest
The authors declare no conflicting interests.

Data Accessibility
All data used in this manuscript are available at this link:
https://figshare.com/articles/Woodlice_cell_size_data/11604063

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Tables

Table 1
Loadings in a principal component analysis (PCA) of cell size in three different cell types from *Porcellio scaber* woodlice reared at two temperatures (15 °C and 22 °C) and two oxygen levels (10% and 22% O₂). PC1 and PC2 scores were used as integrated measures of cell size for hypothesis testing (Table 2).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PC 1</th>
<th>PC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ommatidia</td>
<td>0.56</td>
<td>-0.64</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.51</td>
<td>0.77</td>
</tr>
<tr>
<td>Hindgut epithelium</td>
<td>0.65</td>
<td>-0.06</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>1.86</td>
<td>0.77</td>
</tr>
<tr>
<td>Explained variance</td>
<td>62%</td>
<td>26%</td>
</tr>
</tbody>
</table>
Table 2

Results of four general linear models (GLM) for cell size measures (PC1 and PC2), the magnitude of metabolic increase (MMI) and ommatidia number in *Porcellio scaber* reared at different temperatures (15 °C and 22 °C) and oxygen levels (10% and 22%). PC1 and PC2 were obtained via principal component analysis, and they represent integrated measures of cell size in ommatidia, the hepatopancreas and the hindgut epithelium (see Table 2). The magnitude of metabolic rate increase (MMI) was calculated for each individual woodlouse upon transfer from less to more demanding conditions by dividing the CO₂ consumption rate measured under more demanding conditions (22 °C and 10% O₂) by the CO₂ consumption rate measured under less demanding conditions (15 °C and 22% O₂).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Df</th>
<th>PC1</th>
<th>p</th>
<th>PC2</th>
<th>p</th>
<th>logMMI</th>
<th>p</th>
<th>Ommatidia number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.71</td>
<td>0.40</td>
<td>7.95</td>
<td>0.006</td>
<td>0.17</td>
<td>0.68</td>
<td>2.94</td>
</tr>
<tr>
<td>Body mass</td>
<td>1</td>
<td>295.78</td>
<td>&lt;0.001</td>
<td>2.28</td>
<td>0.13</td>
<td>4.62</td>
<td>0.03</td>
<td>6.62</td>
</tr>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>3.37</td>
<td>0.07</td>
<td>4.42</td>
<td>0.04</td>
<td>1.03</td>
<td>0.31</td>
<td>3.69</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>0.90</td>
<td>0.35</td>
<td>1.57</td>
<td>0.12</td>
<td>0.11</td>
<td>0.75</td>
<td>30.46</td>
</tr>
<tr>
<td>Oxygen x temperature</td>
<td>1</td>
<td>0.74</td>
<td>0.39</td>
<td>2.91</td>
<td>0.004</td>
<td>1.79</td>
<td>0.18</td>
<td>0.33</td>
</tr>
<tr>
<td>Error</td>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 1. Examples of organs of *Porcellio scaber* that were used to assess cell size in individuals reared in two different oxygen levels (10% and 22%) and temperatures (15 °C and 22 °C): A: eye (top view); B: hepatopancreas; C: hindgut epithelium. Abbreviations: BC – B cells; NU – nucleus of epithelial cells; OM – ommatidia. The ring visible on the ommatidium photo is a reflection of light and serves as a tool for orienting a particular ommatidium perpendicularly with respect to the objective.
Fig. 2 The first principal component (PC1) was positively correlated with ommatidia, hepatopancreas cells and hindgut epithelial cells in *Porcellio scaber* (see the loadings in Table 1), and its values increased with increasing body mass. There was a tendency (p=0.07) of PC1 to reach higher values in animals developed under 22% O₂.
Fig. 3. The second principal component (PC2) was positively correlated with the size of hepatopancreas cells and negatively correlated with ommatidia size in *Porcellio scaber* reared at two different oxygen levels (10% and 22%) and two different temperatures (15 °C and 22 °C). A: The values of PC2 differed significantly between animals reared at the two temperatures under normoxia (significant differences are marked with letters) and B: between sexes. On the left side of the graph, the arrows depict loading values of different cell types. The correlation coefficients correspond to the values on the Y axis.
Fig. 4. In Porcellio scaber, a transfer from 15 °C and 22% O₂ (less demanding condition) to 22 °C and 10% O₂ (more demanding condition) resulted in an increase in metabolic rate, but the increase was disproportionally higher among large woodlice than among small woodlice. The magnitude of metabolic increase (MMI) was calculated for each individual woodlouse by dividing the CO₂ consumption rate under more demanding conditions by the CO₂ consumption rate under less demanding conditions. The woodlice were reared under different developmental conditions comprising two oxygen levels (10% and 22%) and two temperatures (15 °C and 22 °C).
Fig. 5. In Porcellio scaber, the number of ommatidia increased with body size. A: Animals developed at high temperature (22 °C) had more ommatidia than individuals developed at low temperature (15 °C), and B: animals developed at normoxia (10% O₂) tended to have more ommatidia than animals developed at hypoxia (22% O₂). C: Females tended to have more ommatidia than males.
Hypoxia causes woodlice (*Porcellio scaber*) to select lower temperatures and impairs their thermal performance and heat tolerance.
Hypoxia causes woodlice (*Porcellio scaber*) to select lower temperatures and impairs their thermal performance and heat tolerance

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Abstract

Environmental temperatures and oxygen availability are important for the balance between oxygen supply and demand. Terrestrial organisms are generally perceived to be less limited by access to oxygen than their aquatic counterparts. Nevertheless, even terrestrial environments can be deficient in oxygen, especially for organisms occurring in soil, litter, wood, rotten fruit or at high elevations. While isopods are the best adapted to a terrestrial lifestyle among crustaceans, many species, including woodlice, occupy environmental gradients of temperature and oxygen. To investigate whether mismatches between oxygen supply and demand can result in a loss of performance in a terrestrial organism, we studied the effects of atmospheric oxygen concentration on the thermal performance of the common rough woodlouse (*Porcellio scaber*). We compared the thermal preference, thermal sensitivity of running speed, and tolerance to extreme temperatures of woodlice exposed to one of two oxygen concentrations (21% - normoxia, 7% - hypoxia). Under hypoxia, *P. scaber* preferred microhabitats with temperatures that were on average 3˚C lower than those preferred under normoxia. The running speed tended to reach its maximum at a lower temperature under hypoxia than under normoxia (25.13˚C vs 28.87˚C, respectively, although p was equal to 0.09), and normoxic woodlice ran approximately 1.5-fold faster than hypoxic woodlice at the point of maximum speed. Heat tolerance was significantly lower under hypoxia (38.9˚C) than under normoxia (40.7˚C), but there was no difference in cold tolerance (5.81˚C under normoxia and 5.44˚C under hypoxia). Overall, our results indicate that environmental gradients of temperature and oxygen may shape the physiological performance of terrestrial ectotherms, likely via their effects on the balance between oxygen supply and demand, which may have fitness consequences for these organisms in nature.

Introduction

Isopods appear to be the most successful land colonizers of all crustaceans [1], and it is estimated that their terrestrial lifestyle evolved independently at least two times [2]. They evolved number of land adaptations, such as pleopodal lungs, a water-conducting system and aggregation and conglobation behaviours [1]. It remains unclear which selective factors drove this
evolutionary transition, but a lower risk of predation and access to oxygen and/or food are likely candidates [3,4]. On the other hand, terrestrial environments can impose new challenges to land colonizers, such as stronger thermal fluctuations combined with an increased risk of desiccation and overheating. Interestingly, the colonization of land by isopods ca. 300 Mya [5,6] coincided with peak levels of atmospheric oxygen, followed by a large (50%) and rapid decrease in the atmospheric oxygen content [6,7]. Therefore, isopod lineages evolving a terrestrial lifestyle experienced dramatic shifts in oxygen availability throughout their evolutionary history: from the relatively low oxygen availability in aquatic environments [8] to the initially high but later decreased oxygen availability on land. Contemporary isopods often inhabit microenvironments in close contact with decomposing organic matter, which are characterized by a lower oxygen supply than the atmosphere. For example, the concentration of oxygen in wet, decaying beech logs (a potential habitat for isopods) can be reduced 40-fold, reaching concentrations as low as 0.5% [9]. Some isopods inhabit periodically submerged sand burrows in intertidal zones, where they can be exposed to hypoxic conditions [10]. Isopods were also reported to inhabit altitudes reaching 4725 m a.s.l. [11], where oxygen pressure can drop to 60% of the pressure at sea level [12].

The oxygen supply is regarded as an important environmental characteristic that has strong fitness consequences because it impacts a myriad of organismal performance metrics, ranging from, e.g., consumption [13], metabolism [10] and growth [14] to behavioural reactions to predators [15]. Nevertheless, an effect of oxygen supply on organismal performance is relative because it strongly depends on the metabolic demand, which is largely determined by the physical and physiological work and body temperature [14]. In principle, the performance of ectotherms is stimulated by warm environments, but as the environmental temperatures approach critical values, their performance rapidly deteriorates, which is ultimately followed by death [16]. Much of the research in the area of thermal performance of ectotherms focuses on the critical temperatures that suppress performance and survival [17–19]. The ability to cope with thermal limits can be governed by the thermal sensitivity of molecules, mainly enzymes [20] and phospholipids [21], but it is also hypothesized to be linked to a temperature-driven imbalance between metabolic supply and demand, which would lead to insufficient oxygen delivered to tissue under thermal extremes [22]. Indeed, upper thermal limits seem to be reduced by hypoxia in many aquatic organisms [23–25], but this phenomenon has been far less studied in terrestrial organisms, which are generally regarded as less often exposed to oxygen deficiency under natural conditions [26]. Nevertheless, some data on lizards [27], insects [28,29] and terrestrial isopods [30,31] suggest that the oxygen supply can affect thermal performance, even in land-dwelling ectotherms, although there is evidence suggesting that thermal limits in terrestrial organisms are affected by only severe hypoxia [32]. The temperature-driven mismatch between oxygen supply and demand also seems to scale up to the level of thermal dependence of the life histories of ectotherms. The thermal environment directly affects ectotherms’ fitness [16] by governing physiological rates [33], predation [34] and mobility [35]. Puzzlingly, most ectotherms mature earlier and reach smaller adult sizes in warmer environments – a pattern often called the temperature-size rule [20]. There is theoretical and empirical evidence that this puzzling pattern is governed by resource allocation to growth and reproduction, which adaptively responds to thermal changes in metabolic supply and demand for oxygen [14,36–39].

Addressing the oxygen-dependent thermal performance of isopods, we performed laboratory experiments on the common rough woodlouse (*Porcellio scaber*). This species of isopod naturally occurs in Europe, excluding south-eastern Europe, and has been introduced to many other continents, such as North America and Australia [40]. *P. scaber* woodlice inhabit decaying leaf litter and logs, so they should naturally occupy an array of microhabitats that differ in
thermal and oxygen conditions. To assess the combined effects of the thermal and oxygen environments on performance, we first performed a choice experiment in which we exposed the studied woodlice to a wide thermal gradient, testing whether their thermal preference undergoes changes with the level of atmospheric oxygen. We hypothesized that woodlice would select cooler microhabitats under hypoxia, decreasing their oxygen demand [41] and/or increasing oxygen affinity of the haemolymph [42,43]. Then, we studied the thermal sensitivity of running speed and thermal physiological limits, testing how oxygen conditions shape these characteristics. Given that a limited access to environmental oxygen should impede aerobic capacity, we predicted that low-oxygen conditions would decrease the maximum level of performance and shift this maximum towards lower temperatures, where decreased demand for oxygen [41] would meet increased oxygen supply associated with the improved oxygen affinity of the haemolymph [42,43]. Since the evidence for oxygen limitation is stronger for heat tolerance than for cold tolerance [44], we expected that the hypoxic woodlice would lower their heat tolerance but tolerate cold extrema equally as well as normoxic woodlice. It is because hypoxia should impose limits especially in combination with increased metabolic demand caused by higher body temperature.

Material and methods
For this study, we collected *P. scaber* in late summer from two monastery gardens (50.059179 N, 19.93604 E; 50.065117 N, 19.931388 E) and from one old backyard (50.070957 N, 19.939061 E) in the vicinity of the Old City of Kraków (Poland). The species is not under protection, and we obtained permission from the landowners to collect it. The animals were maintained at the Institute of Environmental Sciences (Kraków) in a climatically controlled room set to 20˚C and with a 12D:12L photoperiod. Once per week, the animals were provided water and a dry leaf mixture consisting mostly of alder (*Alnus glutinosa*) and ash (*Fraxinus excelsior*). Our experiments included both sexes, and prior to each experiment, the animals were weighed to the nearest 0.001 mg (XP26, Mettler Toledo, Greifensee, Switzerland). In all experiments, we used three thermal platforms connected to an oxygen regulation system (Fig 1). The platforms were built from a one-metre-long metal bar with two Peltier modules on each side (BIOS-PEKT, Kraków, Poland). The modules enabled us to either heat or cool one of the two sides of the bar to obtain a thermal gradient (Experiment 1) or generate a desired temperature for studying thermal performance (Experiments 2 and 3). A platform was enclosed in a transparent Plexiglas cover (YETI, Agencja Reklamy, Kryspinów, Poland), which provided the tested animals with the experimental oxygen concentrations. The concentration of oxygen was continuously monitored by a fuel cell (Sable Systems International, Las Vegas, NV, USA), and hypoxia (7% O₂) was maintained by adding nitrogen (Air Products Sp. z o.o., Kraków, Poland) with a Roxy-4 controller (Sable Systems International), which added gas according to the demand needed to decrease the oxygen level inside the chamber. We used external air to create normoxic conditions. The woodlice involved in all experimental essays were tested on a layer of moist sand (160 ml of water per 500 ml of dry sand; hereafter, moist sand), which provided a semi-natural substrate for the tested animals.

Experiment 1: Thermal preferences
To measure thermal preference, woodlice were placed on a gradient of temperatures generated by the thermal platforms, and each individual woodlouse could freely choose thermal conditions. The Peltier modules on the two ends of each thermal platform were set to either 12˚C or 45˚C to generate a wide thermal gradient. An 8-cm-wide aluminium u-shaped profile was placed on the platform, and its bottom was covered with moist sand. The local temperatures
were recorded in 30-minute intervals (to the nearest 0.1˚C) every 10 cm along the thermal gradient with the help of thermocouples placed in the sand and connected to a computer. After each session, the sand was replaced by fresh sand, which ensured comparable humidity conditions between sessions and allowed us to avoid possible influences of cues left by previously tested animals. During each session, we measured the preferences of three animals, but to ensure independent measures, each animal was placed individually into a narrow corridor. The corridors were produced from 1-cm-wide u-shaped aluminium profiles that ran along the platform. The top of the profile was perforated to ensure air access for the animal. Prior to the measurements, each animal was placed in a random position along the thermal gradient (we used a random number generator) and covered with the profile used to create the corridor. Each measurement session started at 7 p.m. and lasted 12 h. During the sessions, soft light (10% brightness) was provided in the climatically controlled room. In the morning on the next day, the positions of the animals in each corridor were recorded. We measured the distance from each animal to the two nearest thermocouples, which was used to calculate the local temperature at the animal’s position (considered the preferred temperature). The calculation assumed a linear change in the temperatures between the two points where the temperature was measured. In total, we tested 79 animals, both males (34 individuals) and females (45 individuals). On average, each tested animal enclosed in a corridor was exposed to an effective gradient of temperatures that ranged from 16.41˚C to 36.28˚C (as measured directly in the sand at the two ends of the thermal gradient); along this gradient, the temperature changed at an average rate of 0.21˚C per 1 cm.

**Experiment 2: Thermal performance**

To measure the oxygen-dependent thermal performance, the animals were forced to run around a circular glass arena, and we measured their average speed at different temperatures and oxygen levels. The arena was built from a glass container (80 mm in diameter) with a small plastic cylinder in the middle, which created a circular corridor for performing the running assays (Fig 1). Inside this plastic cylinder, another cylinder was placed in which the...
animal was acclimated. To provide the running animals with a semi-natural substrate, the bottom of the arena was covered with a thin layer of moist sand. During measurements, the arena was placed on the thermal platform, immediately above one of the two Peltier modules, which was set to one of 13 different temperatures: 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, or 45˚C. Temperatures were recorded to the nearest 0.1˚C by an iButton (Maxim/Dallas Semiconductor, San Jose, CA, USA) placed in the middle of the arena. The measurements were performed under either hypoxia or normoxia.

Prior to testing, an animal was briefly habituated to the test conditions for 15 minutes in a narrow plastic cylinder located in the middle of the arena used for running tests. During measurements, animals were induced to run by touching the posterior part of the body with a small brush. The brush was manipulated by hand from the outside of the cover that surrounded the thermal platform. To reach the animal with the brush from the outside of the arena without changing the conditions under the cover, the brush penetrated the cover via a flexible material that allowed the entry point to be sealed (Fig 1). The tested animal was placed at a starting point (a line painted on the walls of the arena) and then forced to run. After each lap in the arena (indicated by passing the starting point), the time was recorded on a computer with the help of the estopwatch.net program. The running assay lasted for up to 15 minutes. If an animal did not react to the three touches of the brush during the assay, it was assumed to be exhausted, and the test was ended. For the analysis, we calculated the mean time required to complete one lap in the arena. For logistical reasons, the experiment was run in two fully balanced rounds. For each temperature and oxygen combination, we tested 4 animals (2 males and 2 females). If the temperature measured by the iButton during the assay deviated by 2˚C or more from the desired temperature, the test at that temperature was repeated with other animals, but both results were included in the analysis. In total, we tested 114 animals, both males (56 individuals) and females (58 individuals).

**Experiment 3: Critical temperatures**

To measure oxygen-dependent critical temperatures, we observed changes in the capacity of woodlice to control their body position during exposure to gradual changes (either an increase or a decrease) in temperatures under hypoxia and normoxia. The tested animals were placed in a small plastic container with a metal bottom, and the container was placed on the thermal platform above one of the two Peltier modules. Note that the oxygen conditions were controlled in the same way as in Experiments 1 and 2. The bottom surface of the container was divided into two halves, and each half was dedicated to one animal (two animals were tested simultaneously). To provide a semi-natural substrate for the animals, the bottom of the container was covered with a thin layer of moist sand. The temperature during measurements was recorded to the nearest 0.01˚C by a fast-response thermocouple thermometer (HD 2128.2, Delta OHM, Caselle di Selvazzano, Italy) connected to a computer. The upper critical temperature (CT_{max}) was measured by placing an animal in the test container, allowing the sand temperature to reach 35˚C, and then exposing the animal to a steady increase in temperature at a rate of 0.5˚C per minute. After each 0.5˚C increase, we used a brush to turn the animal over onto its back. If the animal did not regain its position within 30 seconds, the temperature was considered the CT_{max}. For the analysis, the mean of the temperatures recorded every second over this 30-second interval was used as the measure of CT_{max}. In total, we tested 21 animals, both males (10 individuals) and females (11 individuals). The critical minimum temperatures (CT_{min}) were measured by placing an animal in a test container when the sand temperature reached 3.5˚C. After the animal was put into a chill-induced coma, which was confirmed by turning the animals over with a brush and checking if they regained their position within one
minute, we switched off the Peltier module on the thermal platform, allowing the temperature to steadily increase at a rate of 0.5 °C per minute. This rate was determined prior to the measurements. The temperature at which the animal regained its normal position was considered the CT_{min}. In total, we tested 24 animals, both males (12 individuals) and females (12 individuals).

**Statistical analysis**

The analysis was performed in R 3.4.1 software [45] with the help of the nlme [46], ggplot2 [47] and effects [48] packages. The data on preferred temperatures and critical temperatures (CT_{max} and CT_{min}) were analysed with a general linear model (GLM) including sex and oxygen conditions (with an interaction term) as grouping predictors and body mass as a covariate. The running speed data were analysed with a nonlinear mixed model (nlme function). The fixed parts of the model were sex as a grouping predictor and temperature and body mass (without interactions) as numeric predictors. Following Lachenicht et al. [49], we assumed that the thermal dependence of the running speed, our thermal performance curve, took the shape of a 3rd-order polynomial function. The random parts of the model were the experimental round as well as the random estimates of our thermal performance curve parameters. To test if the effect of the experimental round was significant, we ran a similar model without the random effect (using the lm function). We compared the Akaike information criterion (AIC) values of these models and chose the best model as that with the lowest AIC.

To examine whether normoxia and hypoxia were characterized by different maximal performances (MPs) and temperatures at which these maxima were achieved (T_{MP}), we designed a simplified version of the GLM that considered only oxygen and temperature as fixed factors. In the first step, we estimated parameters of the fitted thermal performance curve (3rd polynomial) for normoxia and for hypoxia. After differentiation of these functions, we computed the point at which the first derivative of each function reached zero, which corresponded to finding the MP and T_{MP}. In the second step, we calculated differences in MP and T_{MP} between the oxygen treatments. To test whether these differences were statistically significant, we used an approximate randomization test [50], which compared the observed differences in MP and in T_{MP} with a distribution of randomly generated differences in MP and in T_{MP}. An observed difference was regarded significant when its value occurred among 5% of the rarest randomly generated values. The distribution of randomly generated differences was produced via 10000 randomizations. Each randomization involved i) pooling the data on the temperature dependence of running speed in our two oxygen treatments, ii) randomly re-assigning these data to our oxygen treatments, iii) computing the MP and T_{MP} for the two (normoxia and hypoxia) randomly generated thermal performance curves (we used the same statistical and mathematical tools that were used to calculate the observed values of MP and T_{MP}), and iv) computing the difference in MP and in T_{MP} between the two randomly generated curves.

Above 37.59°C, some animals died during our performance tests, and the data for these cases were not used in the computation of thermal performance curves. Nevertheless, we decided to further explore these mortality data in a separate analysis that addressed whether the risk of mortality during our tests depended on oxygen conditions. We used a chi-square test that compared the proportion of dead animals between the normoxia and hypoxia conditions in tests carried out above 37.59°C.

**Results**

Compared to woodlice exposed to normoxic conditions, woodlice exposed to hypoxia selected microsites with lower temperatures (F_{1,74} = 11.66, p = 0.001); on average, the preferred
temperatures under hypoxia and normoxia were 17.68˚C and 20.93˚C (Fig 2), respectively.
The sex of woodlice (F
1,74
= 0.50, p = 0.48), the sex x oxygen interaction (F
1,74
= 1, p = 0.32), and the body mass of woodlice (F
1,74
= 0.31, p = 0.58) did not affect temperature selection.

In the analysis of the thermal sensitivity of running speed, the model without a random
effect had a lower AIC score than that with the random effect; consequently, the results of this

Fig 2. Common rough woodlice preferred lower temperatures when exposed to oxygen deficiency (p = 0.001).
Means ± 95% CIs from the general linear model with body mass as a covariate and sex as an additional factor.

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best model are presented here. Sex ($F_{1,84} = 1.65, p = 0.20$) and body mass ($F_{1,84} = 0.11, p = 0.75$) did not affect the performance of woodlice. Two parameters of the performance curve differed between hypoxia and normoxia (intercept: $F_{1,84} = 14.47, p = 0.001$; linear coefficient: $F_{1,84} = 6.3, p = 0.01$), and two were not different (quadratic coefficient: $F_{1,84} = 2.21, p = 0.14$; cubic coefficient: $F_{1,84} = 1.83, p = 0.18$). The temperature at which maximal performance was reached ($T_{\text{MP}}$) was 25.12˚C under hypoxia and 28.87˚C under normoxia (Fig 3). Our randomization test showed that the difference between these temperatures was almost significant ($p = 0.09$; S1 Fig). The maximal performance at these temperatures (MP) was 0.044 laps per sec under hypoxia and 0.069 laps per sec under normoxia, and this difference was significant ($p < 0.001$; S2 Fig). Compared to normoxic conditions, hypoxia resulted in an increased proportion of animals that did not survive the performance tests at temperatures above 37.59˚C ($\chi^2 = 6.13, p = 0.01$), indicating that hypoxic conditions lowered the tolerance of woodlice to high temperatures. In the analysis of critical temperatures, we found that animals exposed to hypoxia had a lower $CT_{\text{max}}$ (38.90˚C) than animals exposed to normoxia (40.66˚C) ($F_{1,16} = 6.41, p = 0.02$, Fig 4). Sex ($F_{1,16} = 0.02, p = 0.88$), the sex x oxygen interaction ($F_{1,16} = 2.50, p = 0.13$), and body mass ($F_{1,16} = 0.07, p = 0.80$) did not affect $CT_{\text{max}}$. In contrast, the value of $CT_{\text{min}}$ (5.81˚C under normoxia and 5.44˚C under hypoxia, Fig 4) was not affected by oxygen ($F_{1,19} = 0.67, p = 0.42$), sex ($F_{1,19} = 0.09, p = 0.76$), the sex x oxygen interaction ($F_{1,19} = 0.67, p = 0.42$) or body mass ($F_{1,19} = 0.15, p = 0.70$).

Discussion

Our experimental results indicate that when exposed to hypoxic conditions, *P. scaber* chooses to occupy microsites with low temperatures. In nature, woodlice inhabit sites with different oxygen conditions [10], so our evidence suggests that isopods exposed to poor oxygen conditions, e.g., those due to rapid decomposition or high altitude, might prefer to stay in cooler sites. The links between oxygen availability and preferred temperatures have rarely been studied, but the available evidence shows that oxygen deprivation also decreases preferred temperatures in some species of protists [42], fish [43,51], amphibians, reptiles [41], and even
mammals [42]. This effect of hypoxia on the choice of thermal environments can be explained by a number of different mechanisms, depending on the biology and ecology of the tested species. For example, in ectotherms, selecting cooler microhabitats will lower physiological rates and hence oxygen demand [41]. In animals with oxygen-binding metalloproteins, such as the haemocyanin in isopods, lower body temperature increases blood affinity to oxygen [42,43]. In actively breathing animals, lower temperatures might also decrease costs of ventilation [42,52], but this mechanism is not relevant for isopods, which ventilate passively. Importantly, in contrast to our findings in *P. scaber* woodlice, hypoxic conditions did not change the temperatures preferred by tarantula spiders [42]. This is somewhat surprising because in principle, arachnids and isopods have similar oxygen-delivery systems that involve a gas-exchange organ that exchanges gases with the ambient air (book lungs in spiders and pleopodal lungs in isopods) and a circulating haemolymph system that delivers oxygen to tissue with the help of oxygen-binding protein. On the other hand, isopods and spiders are distantly related groups, and many other mechanisms could account for this difference in the response to hypoxia.

We found a negative effect of hypoxia on the $CT_{\text{max}}$ of *P. scaber* (measured in two different ways: heat coma and survival at high temperatures), which indicates that the upper thermal limits of terrestrial crustaceans can change with oxygen conditions in the environment. The two-stage gas-exchange system of isopods might be expected to increase the hypoxia sensitivity of thermal performance compared to the one-stage tracheal system of insects [30] because the...
The affinity of haemocyanin to oxygen decreases with an increase in temperature [44]. The tracheal system delivers oxygen directly to insect tissues and lacks specialized carriers of oxygen [10], but binding proteins are used by some insects for oxygen storage [53]. Klok et al. [30] and Stevens et al. [31] compared the CT$_{max}$ of isopods and insects by measuring the metabolic rate and examining their activity. In both studies, isopods (either P. scaber or Armadillidium vulgare) decreased their CT$_{max}$ under hypoxia, in agreement with our results. Klok et al. [30] also showed that a beetle (Gonocephalum simplex) tolerated hypoxia well and did not change its CT$_{max}$ (activity was decreased by only a decrease in the O$_2$ level to 2.5%). Stevens et al. [31] reported that under hypoxia, a beetle (Tenebrio molitor) exhibited a decrease in CT$_{max}$ of 6.9°C compared to an isopod, which decreased its CT$_{max}$ by 10.6°C. Importantly, Stevens et al. [31] also examined the effects of hypoxia on cold tolerance (CT$_{min}$), concluding that cold tolerance was affected by oxygen conditions in neither isopods nor insects. This conclusion also agrees with our evidence for P. scaber, which shows no effect of oxygen conditions on the tolerance of low temperatures. Oxygen limitation likely decreases in the cold as a result of a dramatic decrease in the rate of metabolism and thus in the demand for oxygen.

In the same thermal habitat, our experimental woodlice were able to run at a maximum of ca. 1.5 times faster under normoxia than under hypoxia, which suggests that the ability of P. scaber to perform highly metabolically demanding tasks can be limited by the oxygen supply in the air. The temperature at which the speed reached its maximum was lower under hypoxia (25.13°C) than under normoxia (28.87°C), although this difference was only nearly statistically significant. Thermal performance curves have rarely been studied in isopods, especially in the context of oxygen limitation, so we do not have many empirical references for evaluating whether this pattern is generally observed in P. scaber or other isopods. The evidence available in the literature indicates that running isopods achieve their highest speed at temperatures higher than those that maximized running speed in our experiment (ca. 25-29°C). For example, Dailey et al. [35] observed a continuous increase in the running speed of Porcellio laevis at temperatures approaching 35°C, but temperatures higher than 35°C were not tested; therefore, it is difficult to conclude the exact temperatures that maximize the performance of this species. Schuler et al. [54] observed that P. scaber reached its maximum speed in the range of 33-34°C, which was much higher than the temperature in our running essays. Certainly, the results of comparisons of nominal trait values between studies that involved different populations and methods should be interpreted with caution. For example, we forced woodlice to run for 15 minutes or until complete exhaustion, but the previous studies used much shorter observation times. Therefore, it is likely that instantaneous performance is maximized by higher body temperatures than is sustainable performance. If so, our results suggest that hypoxic conditions are the limiting factor for isopods involved in prolonged locomotor activity, but future studies should evaluate whether their instantaneous performance is also oxygen-sensitive.

Mobility has different selective advantages, including the capacity for behavioural thermoregulation, foraging, and predation avoidance [35]. Is not clear how well the ability of isopods to achieve high running speeds correlates with their predation avoidance or with other fitness-related consequences. According to Sunderland et al. [55], isopods might rarely be attacked by predators, and most such attacks are directed towards juveniles. However, some predators are believed to be isopod specialists, such as the woodlouse spider Dysdera crocata (but see [56]). The preferred temperatures of D. crocata [57] appear to closely match the preferred temperatures of its potential prey, the sympatric woodlouse P. laevis [58]. Other studies demonstrated that the presence of predator cues increased turn alternations in isopods [59]. Moreover, if spiders prove to be less oxygen-limited than isopods (as speculated in the first paragraph of our Discussion), then woodlouse spiders would not change their predation intensity on isopods in oxygen-deficient microhabitats, which would result in increased predation pressure on the
oxygen-limited woodlice, causing them to become easy prey in hypoxic habitats. This scenario certainly requires rigorous testing.

Integrating our data on thermal preferences and performance, we found a notable mismatch between the temperatures preferred by woodlice and the temperatures at which woodlice achieved their peak locomotor performance: the preferred temperature was lower than the peak-performance temperature. Such mismatches might indicate a conflict between the habitat preferences of resting organisms that must involve decisions about energy expenditure and long-term fitness consequences and the thermal sensitivity of active organisms that must involve the thermal dependence of muscle physiology. Interestingly, the mismatch between the preferred and peak-performance temperatures was slightly larger under normoxia (a difference of 7.94°C) than under hypoxia (a difference of 7.45°C). Note here that the preferred temperatures were more strongly affected by oxygen availability than were the temperatures that ensured peak performance. According to Portner [60], aerobic scope (the difference between minimal and maximal metabolism) is a factor that integrates many physiological and ecological processes (immunological, behavioural, growth, foraging, etc.). Aerobic scope is expected to reach its maximum at an optimal temperature, decrease towards the thermal limits and approach zero at critical temperatures. Thus, a single optimal temperature is predicted for all physiological processes (the model described with reference to fish) [60,61]. Our evidence for the mismatch between the preferred temperatures and peak-performance temperatures is not consistent with such a single optimum temperature. Clark et al. [61] suggested that different types of performance should follow different thermal sensitivities with different optima rather than one universal pattern. From a broader life-history perspective, it is hard to imagine that the temperatures that maximize a given type of physiological performance are evolutionarily adaptive (e.g., an ectotherm that maintains a body temperature that maximizes locomotor performance does not necessarily maximize its expected lifetime reproductive output) only because different measures of performance and fitness play out on different timescales.

Overall, the results of our study provide important insight into the ecologically relevant consequences of micro-environmental gradients in temperature and oxygen availability. Importantly, we focused on multiple elements of thermal performance (thermal preferences, thermal limits, and thermal sensitivity of mobility), which helped us develop an integrated view of how differences in microhabitat temperature and oxygen availability might affect terrestrial isopods in nature. The detected mismatch between the temperatures that were preferred by woodlice and the peak-performance temperatures indicates that attempts to draw simplifying inferences about species-specific ecological optima should be made with caution. From a larger perspective, our results can help address how the oxygen sensitivity of thermal performance shapes the geographic distribution of terrestrial isopods and their expected responses to global climate change. It was already demonstrated that P. laevis adapts to local thermal conditions along latitudes, showing sharp latitudinal clines in thermal optima, thermal performance and thermal tolerance [58]. Additionally, species of isopods have evolved different respiratory organ anatomies, which likely reflect specializations to conditions of varying humidity [62,63]; however, these adaptations should also have consequences for oxygen deprivation tolerance [10].

Supporting information

S1 Fig. Distribution of randomly generated differences in the temperature at which the maximal performance (running speed) of common rough woodlice (Porcellio scaber) was achieved (T_{MP}) between two oxygen treatments (normoxia and hypoxia). The distribution was obtained via 10000 randomizations (see Material and methods). The empirical difference
in $T_{\text{MP}}$ calculated from the original data is indicated by the red line.

(TIF)

S2 Fig. Distribution of randomly generated differences in the maximal performance (MP, measured as running speed) of common rough woodlice (*Porcellio scaber*) between two oxygen treatments (normoxia and hypoxia). The distribution was obtained via 10000 randomizations as described in detail in the Material and methods section. The empirical difference in MP calculated from the original data is indicated by the blue line.

(TIF)

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Formal analysis: Andrzej Antöl.

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Writing – original draft: Andrzej Antöl.

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References


Supplementary material

Hypoxia causes woodlice (*Porcellio scaber*) to select lower temperatures and impairs their thermal performance and heat tolerance

Andrzej Antol, Wiktoria Rojek, Sanjeev Singh, Damian Piekarski, Marcin Czarnoleski

**Fig. S1** Distribution of randomly generated differences in the temperature at which the maximal performance (running speed) of common rough woodlice (*Porcellio scaber*) was achieved ($T_{MP}$) between two oxygen treatments (normoxia and hypoxia). The distribution was obtained via 10000 randomizations (see Material and methods). The empirical difference in $T_{MP}$ calculated from the original data is indicated by the red line.

**Fig. S2** Distribution of randomly generated differences in the maximal performance (MP, measured as running speed) of common rough woodlice (*Porcellio scaber*) between two oxygen treatments (normoxia and hypoxia). The distribution was obtained via 10000 randomizations as described in detail in the Material and methods section. The empirical difference in MP calculated from the original data is indicated by the blue line.
General discussion

In my research I studied different phenomena with a help of various theoretical approaches but ultimately my results can be viewed in a perspective of the life history evolution theory. It is because the holistic view of an organism provided by the life history theory gives the opportunity to see the organism as it is ‘seen by selection’. I mean here an organism as an organic machine which changes acquired resources to itself or its offspring body and the final score of this machine function is the number of fertile offspring released, which we call fitness. My research should be seen in this perspective because all specific questions and predictions that I considered can be ultimately reduced to the question of how a particular character helps to maximize fitness.

The basic life history trait I investigated in *P. scaber* with reference to other isopod species was offspring size and how it is shaped by female size (Study I). According to theories that predict an impact of brooding on life history strategy (Heino and Kaitala, 1996; Jørgensen et al., 2011), I expected that due to higher survivability, bigger females would be able to produce bigger offspring, which would cause a positive offspring size-female size correlation (Jørgensen et al., 2011). My second expectation was that the costs of brooding (either decreased physiological performance or increased mortality of females) would favor indeterminate growth strategy in isopods, which would be manifested by a high difference in body size among the smallest and the largest reproducing *P. scaber* females (an indicator of the intense postmaturation growth). My results suggest that brooding might indeed have an important role for shaping life histories of isopods, leading to the prevalence of indeterminate growth in this taxon and the origin of a correlation between offspring size and female size. Importantly, the origin of this correlation received weaker support from my data for *P. scaber* compared to the origin of indeterminate growth. I found that in *P. scaber* bigger mothers produced bigger offspring only under certain conditions, when their clutches were extremely small, independently of their (mothers’) body size. Unfortunately, my review revealed that there is still little data on offspring size in other species of isopods. Nevertheless, available published evidence suggests that in approximately half of studied isopod species, bigger mothers produce bigger offspring. I hope that my results will stimulate future studies of this phenomenon, which would help to formulate a wider evolutionary view of isopods’ life histories.

Indeterminate growth was found to characterize many isopod species and my results also support the existence of this growth strategy in *P. scaber*: interestingly, I found approximately six fold differences in body mass between the smallest and the biggest
reproducing females of *P. scaber*. Interesting results have been published by Hariharan et al. (2016) who used phylogenetic data to propose that indeterminate growth might be an ancestral state in the evolution of growth patterns. The prevalence of indeterminate growth strategy in nature is regarded as a puzzle (Heino and Kaitala, 1996), but costs associated with brooding may put a light on why it is so widespread in extant species. In this view, brooding with its selective pressure for the origin and maintenance of indeterminate growth can be considered as an important factor in isopods’ evolution which limits the evolution of determinate from indeterminate growth pattern over time.

As I mentioned in the Introduction, following their land invasion, isopods were undergoing evolution under a dropping atmospheric oxygen level (Berner et al., 2007; Broly et al., 2013). It may have set an important selective pressure, leading to adaptations that help to cope with decreasing oxygen availability. As investigated in other study (Horváthová et al., 2017), lengths of different phases (aqueous and gaseous) of offspring development in brood pouch differs between normoxic and hypoxic conditions. This might be the consequence of evolution in low oxygen and the mean to deal with these conditions. Following this perspective, my further research (*studies II-IV*) explored how performance of *P. scaber* was shaped by two important ecological factors: temperature and oxygen. I found that these factors had the highest effect on growth (temperature; *Study II*) and physiological performance (temperature and oxygen; *Study IV*) of *P. scaber*, but they had much less pronounced effect on cell size (oxygen; *Study III*).

In **Study II**, I investigated how temperature and oxygen affected the growth and size of gas-exchange organs in *P. scaber*. I expected that development in different temperatures would lead to growth responses in accordance with Temperature Size Rule (TSR) (Atkinson, 1994). Indeed, I found faster development to smaller body size in *P. scaber* exposed to high temperatures, but a difference in asymptotic size between two temperatures was small. What is surprising, I did not find any effects of oxygen on growth pattern. It is contrary to previous results for rotifers (Walczyńska et al., 2015) or a freshwater isopod Asellus aquaticus (Hoefnagel and Verberk, 2015), which suggested that TSR could be driven by oxygen deficiency. In **Study III**, I focused on cell size and metabolic performance and in line with the Theory of Optimal Cell Size (TOCS) I expected adaptive plastic changes in cell size in the response to different thermal and oxygen conditions. TOCS predicts that either hypoxia or higher temperature should support development of smaller cells in order to maintain efficient oxygen transportation to tissue. In my research, I found weak evidence to support this hypothesis (statistical tendency to develop smaller cells by animals in hypoxia and no effect of developmental temperatures on cell size). There was also no effect of rearing
conditions on the ability to metabolically deal with demanding conditions, but in fact, this effect conforms with TOCS: animals that did not respond to rearing conditions by cell size changes should not differ in their physiological performance.

It is interesting to interpret my results of no effect of oxygen conditions on growth pattern (Study II) and a small effect of oxygen conditions on cell size (Study III) with consideration of my data on the plastic changes of lung size in response to developmental conditions (Study II). These results demonstrated that *P. scaber* could modify the lungs size according to oxygen and thermal conditions during development as well as according to oxygen requirements at different life stages. I hypothesize that this might be the reason why woodlice did not differ in growth rate between oxygen conditions. It is also likely that the compensation of oxygen delivery caused by changes in lung size could reduce the necessity of drastic changes in cell size. It is worth mentioning that a factor which I did not take into account, but which may play a role in shaping lung size, is water saving, as isopods lose water mainly by lungs and cuticle (Warburg, 1993). This view is discussed with more detail in the Study II.

Developmental phenotypic reactions to living in oxygen deficient environment are likely to indicate plastic responses that resemble ecological situations of permanent living under certain conditions (e.g., inhabiting oxygen deficient habitats like decaying wood logs), whereas responses to acute hypoxia can indicate capacity to respond to short-term oxygen fluctuations (e.g., an inundation during flood, a shift in decomposition rate, or moving to locally hypoxic/normoxic sites). Intriguingly, my study showed much more profound effect of severe oxygen deficiency on *P. scaber* in acute assays (Study IV) than in a long term experiment (studies II & III). The results of Study IV showed that, according to predictions, lower oxygen level led to lower preferred temperatures, lower maximum of performance curve (also the maximum shifted towards lower temperatures) and lower maximal critical temperature. The effect of oxygen on critical temperature accords with predictions of the Oxygen and Capacity Limited Thermal Tolerance (OCLTT) theory (Pörtner, 2001), which explains the maximal thermal limits by the mismatch between oxygen delivery to tissues and oxygen demand (which increases due to high metabolism in high temperatures). It should be stressed here that the effects of oxygen which I found on thermal performance and thermal preference are not directly inferred from OCLTT, and these findings enlarge the view of oxygen limitation in ectotherms and help to understand the interaction between oxygen and temperature in a wider perspective. Additionally, I found arguments in favor of the idea that different physiological traits can have different thermal optima (Clark et al., 2013) as in my research the preferred temperature was lower from the temperature that
maximized the running speed of *P. scaber*. I suggest that these results may be interpreted not only mechanistically as ways to explain thermal limits, but also can help to understand ecological factors affecting reproduction, food acquisition and predation in isopods inhabiting natural conditions.

To summarize the effects of oxygen and temperature, I suggest that oxygen strongly limits thermal biology of isopods in short-term time scale (minutes, hours) but has much weaker effect on isopods in a long-term time scale (lifetime). It means that in longer time scale developmental plasticity may mask the effect of oxygen on growth or cell size. It may be the reflection of permanent oxygen deficiency in natural habitats. I found a weak support in *P. scaber* data for TOCS, which suggests that we should consider that cell size may be shaped by different factors and the effect of temperature and oxygen may be not enough to explain the variance of this trait in animals. Certainly, it is also likely that using other cell types and perhaps using lower oxygen levels might lead to more profound differences in cell size (note that in the long term experiment the oxygen level was set to 10%, but in performance experiments it was set to 7%). Also I found no effect of oxygen on growth pattern and one of possible factors masking effects of oxygen on either growth or cell size might be developmental plasticity of *P. scaber* in lung size. Some of my data support OCLTT, namely common rough woodlice reduced their maximal critical temperature in hypoxia. However, one of OCLTT predictions, about a universal optimal temperature, was not supported by my data, as I found differences between preferred temperatures and the temperature in which performance curve reached its maximum.

**Future prospects**

My results can promote further research on isopods, especially in the area of acute hypoxia responses in different isopod species, which differ in desiccation tolerance according to lung type (Hoese, 1982; Hsia et al., 2013; Schmidt and Wägele, 2001) and cuticle thickness (Csonka et al., 2013). Lungs that are more isolated from external air are present in dry adapted species as an adaptation to limit water loss through respiratory tissue (Hoese, 1982; Hsia et al., 2013; Schmidt and Wägele, 2001). This raises an intriguing possibility that the isolation of lung respiratory tissue may lead to lower performance of isopods under hypoxic conditions. I suspect a presence of a trade-off between humidity tolerance and hypoxia tolerance caused by a limited access to atmospheric oxygen of isolated lungs, which reduce evaporative loss of water but limit intake of air oxygen. This effect can have a consequence for hypoxia tolerance, thermal preference and other isopods’ traits. Understanding this potential tradeoff may lead us to the enrichment of knowledge on land
colonization mechanisms in isopods. The knowledge on lungs could be enlarged by looking into the changes in respiratory area in different species characterized by different lung types in long term studies with manipulation of oxygen, temperature or humidity. In such experiments, it would be beneficial to use more direct techniques to assess the size of respiratory area (not only lung size as a proxy). Another possibility which arises from my research on lung size (Study II) and needs further investigation is the actual significance of pleopodal lungs in oxygen delivery. We know that isopods can breathe not only via lungs but also through cuticle (Edney and Spencer, 1955) and the amount of air acquired by this mean differs between species. Unfortunately, there are no experimental studies in this field. Also, it would be valuable and novel to study respiration rates of different isopod species with and without blocked access of air to pleopods to see if cuticle respiration can significantly help in oxygen delivery in hypoxia or different humidity levels. In a larger scale, gaining insight in this phenomenon may help to explain the ranges of geographic distribution of different isopod species as well as future alternations to these ranges caused by global climate changes. My results also suggest the need of further investigations of isopods focused on mothers’ investments in offspring. This research should include theoretical modeling of evolutionary phenomena that drive the origin of a dependence between mother size, offspring size and offspring number.

Conclusions

Overall, I found significant effects of oxygen, temperature and female body mass on *P. scaber* biology. These effects were visible as changes in cell size, life history traits and physiological performance. The effect of oxygen was especially pronounced when animals were exposed to acute hypoxic conditions which may resemble the sites they occupy. Under chronic hypoxia, the effect of oxygen was visible only for lung size and the effect of temperature for growth rate. My results suggest that isopods evolved adaptations to survive in chronic oxygen deficiency and plastic changes of lungs may be the key factor here. Evolution driven by chronic hypoxia may be caused by permanent occupation of oxygen deficient habitats or by a drastic drop in oxygen availability which happened just after land colonization by isopods. Although my research mainly concerned *P. scaber*, it offered me an opportunity to formulate three generalizations about isopods, which have apparent relevance for isopods’ ecology:

1. Brooding in isopods may be an important evolutionary factor that leads to the origin of a positive correlation between offspring size and mother size and favors indeterminate growth strategy.
2. Thermal and oxygen conditions during development should be regarded as two important environmental factors that shape isopods’ traits, with temperature affecting mainly lung size and somatic growth, and oxygen level affecting mainly lung size and to small extend cell size.

3. Acute changes of oxygen conditions should be regarded as an important factor that shapes isopods’ thermal performance and thermal tolerance as well as their preferences for thermal conditions in microhabitats.
References


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Mgr Andrzej Antoł czynny brał udział w dyskusjach nad koncepcją badań, wnosił swoje pomysły, zwłaszcza zrobienie pracy przeglądowej. Zebrał i zanalizował wszystkie dane, napisał pierwszą wersję manuskryptu i prowadził prace nad dalszymi wersjami.

Oświadczam, że powyższej zamieszczone informacje, a zwłaszcza mój udział w przygotowaniu ww. manuskryptu jest zgodny ze stanem faktycznym:

mgr Andrzej Antoł – Autɔ, Autɔ 12.01.2020

dr hab. Marcin Czarnoleski, prof. UJ – 27.01.2020 Czarnoleski

Thermal and oxygen conditions during development cause common rough woodlice (*Porcellio scaber*) to alter the size of their gas-exchange organs. Praca wysłana do Journal of Thermal Biology

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Mgr Andrzej Antoł brał czynny udział w dyskusjach nad koncepcją i znaczeniem badań oraz wniósł swoje pomysły. Zajmował się eksperymentem (zbieranie zwierząt, karmienie, utrzymanie wilgotności oraz obsługa techniczna) przez cały czas jego trwania. Przy użyciu stworzonego na potrzeby badań programu komputerowego dokonał pomiarów wielkości płuc. Przeprowadził wszystkie analizy statystyczne, napisał pierwszą wersję manuskryptu i prowadził prace nad jego kolejnymi wersjami.

Oświadczam, że informacje zamieszczone powyżej, a zwłaszcza mój udział w przygotowaniu ww. manuskryptu jest zgodny ze stanem faktycznym:

mgr Andrzej Antoł – Andrz Ant 21.01.2020

dr Anna Maria Łabęcka – Anna Marie Labieck 22.01.2020

dr Terézia Horváthová –

dr Bartosz Zieleński –

mgr Natalia Szabla – Natalia Szabla 23.01.2020

mgr Yaroslav Vasko – Yaroslav 23.01.2020

dr hab. Anna Pecio, prof. UJ –

dr hab. Marcin Czarnoleski, prof. UJ –

prof. dr hab. Jan Kozłowski –

24.01.2020
Oświadczenie o procentowym udziale w przygotowaniu publikacji wchodzącej w skład rozprawy doktorskiej magistra Andrzeja Antoła


Oświadczam, że mój udział w przygotowaniu ww. artykułu kształtował się w następujący sposób: 8% przeprowadzenie eksperymentów, 3% przygotowanie manuskryptu, całościowy udział oceniam na 2.75%.

dr Terézia Horváthová

<table>
<thead>
<tr>
<th>Autor</th>
<th>Koncepcja badań [%]</th>
<th>Zbieranie danych [%]</th>
<th>Analiza i interpretacja [%]</th>
<th>Przygotowanie manuskryptu [%]</th>
<th>Całkowity wkład [%]</th>
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<td>Marcin Czarnołęski</td>
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</tbody>
</table>

Mgr Andrzej Antol brał udział w dyskusjach nad koncepcją badań. Zajmował się eksperymentem (zebranie zwierząt, karmienie, utrzymanie wilgotności oraz obsługa techniczna) przez cały czas jego trwania. Przeprowadził pomiary respiracji, fotografował i zmierzył wszystkie rodzaje komórek. Przeprowadził wszystkie analizy statystyczne, napisał pierwszą wersję manuskryptu i prowadził prace nad jego kolejnymi wersjami.

Oświadczam, że powyższej zamieszczone informacje, a zwłaszcza mój udział w przygotowaniu ww. manuskryptu jest zgodny ze stanem faktycznym:

mgr Andrzej Antol –  

dr Anna Maria Łabęcka –  Anna Maria Łabęcka 22.01.2020

dr Terézia Horváthová –

gmr Anna Sikorska – Anna Sikorska 22.01.2020

gmr Natalia Szabla – Natalia Szabla 23.01.2020

dr hab. Ulf Bauchinger –

dr hab. Marcin Czarnołęski, prof. UJ –

prof. dr hab. Jan Kozłowski – 24.01.2020

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Oświadczenie o procentowym udziale w przygotowaniu publikacji wchodzącej w skład rozprawy doktorskiej magistra Andrzeja Antoła


Oświadczam, że mój udział w przygotowaniu ww. artykułu kształtował się w następujący sposób: 10% przeprowadzenie eksperymentów, 2% przygotowanie manuskryptu, całościowy udział oceniam na 3%.

[Signature]

dr Terézia Horváthová

Institute of Soil Biology, Biology Centre CAS
Na Sadkach 7
37005 Ceske Budejovice
Czech Republic

<table>
<thead>
<tr>
<th>Autor</th>
<th>Koncepcja badań [%]</th>
<th>Zbieranie danych [%]</th>
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<th>Przygotowanie manuskryptu [%]</th>
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Oświadczam, że informacje zamieszczone w powyższej tabeli, a zwłaszcza mój udział w przygotowaniu ww. manuskryptu jest zgodny ze stanem faktycznym:

mgr Andrzej Antol – 22.01.2020

mgr Wiktoria Rojek – Wiktoria Rojek 22.01.2020

mgr Sanjeev Singh – Sanjeev 14/1/2020

Damian Piekarski – Damian 24.01.2020

dr hab. Marcin Czarnołęski, prof. UJ – 22.01.2020
W związku z wyjazdem zagranicznym współautora mgr Sanjeev Singh przed uzupełnieniem tabeli procentowego udziału współautorów, przesyłam poniższe oświadczenie: