

Blood as fuel: the metabolic cost of pedestrian locomotion in *Rhodnius prolixus*

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Summary statement:

Locomotion generates an increase in the metabolic rate of haematophagous bugs and changes the respiratory pattern. The energetic cost of pedestrian locomotion allows estimating maximal walking dispersion in Chagas vectors.

ABSTRACT

Active searching for vertebrate blood is a necessary activity for haematophagous insects, and it can be assumed that this search should also be costly in terms of energetic expenditure. Either if it is by swimming, walking, running or flying, active movement requires energy, increasing metabolic rates relative to resting situations. We analysed the respiratory pattern and the energetic cost of pedestrian locomotion in the blood-sucking bug *Rhodnius prolixus* using flow-through respirometry, by measuring carbon dioxide emission and water loss before, during and after walking. We observed an increase of up to 1.7-fold in the metabolic rate during walking as compared to resting in male *R. prolixus* and 1.5-fold in females, as well as a change in their respiratory pattern. The last switched from cyclic during resting to continuous, when the insects started to walk, remaining this condition unchanged during locomotion and for several minutes after stopping. Walking induced a significant loss of weight in both, males and females. This can be explained by the increase in both, the metabolic rate and the water loss during walking. These data constitute the first metabolic measures of active hematophagous insects and provide the first insights on the energetic expenditure associated to the active search for blood in this group.

INTRODUCTION

Resource self-procuring requires active search for most animals. For limbed animals, locomotion is the mechanism that assures this task. Either if it is by swimming, walking, running or flying, active movement requires energy expenditure, which induce a measurable increase in metabolic rate, respect to resting periods.

The amount of energy necessary for self-transporting depends on several factors. For terrestrial locomotion, the weight of the animal, the distance of displacement and the speed of movement should be considered as suitable bases for measuring and comparison (Herreid, 1981; Schmidt-Nielsen, 1972; Tucker, 1970). Full (1989) found a remarkable similarity between data of metabolic cost of terrestrial locomotion for different arthropods and larger animals as lizards, birds and mammals. As the energetic cost of transport is directly proportional to the metabolic rate, which in turn depends directly of the rate of oxygen consumption, assessing the exchange of respiratory gases in an animal, appears as an adequate way to estimate energy costs of locomotion.

Respiratory metabolism and energetics during terrestrial locomotion in invertebrates such as insects have been relatively less studied over the last 30 years in comparison to vertebrates (Lipp et al., 2005). Most studies on insects have focused on energetics of horizontal running (Bartholomew et al., 1985; Bartholomew et al., 1988; Duncan and Lighton, 1997; Herreid et al., 1981; Jensen and Holm-Jensen, 1980; Lehmann and Schutzner, 2010; Rogowitz and Chappell, 2000) and climbing slopes (Full and Tullis, 1990; Lipp et al., 2005), as well as load carriage (Bartholomew et al., 1988; Schilman and Roces, 2005). Few studies, however, assessed the metabolic rate and energetic expenditure during voluntary walking (Berrigan and Lighton, 1994; Lipp et al., 2005; Rothe and Nachtigall, 1989).

Metabolic rate during pedestrian locomotion is probably much lower than during flight. However, metabolic costs of pedestrian locomotion could reach an important portion of the total energy budget associated to flight, particularly in species that search for food or explore potential substrates for oviposition by walking (Berrigan and Lighton, 1994; Full, 2010).

For hematophagous insects, starvation appears to be a main cause of locomotion initiation (Lehane, 2005; Lehane and Schofield, 1982; McEwen and Lehane, 1993). In

the case of disease vectors, this association between locomotion and host-search for feeding acquires particular relevance for the spread of transmitted pathogens. Yet, there is at present no information available relative to energetic cost of locomotion in blood-feeding insects.

One way of evaluating the cost associated to self-transport, is comparing the metabolism of an organism at resting and during locomotion. Metabolic activity has been successfully measured in different species of blood-sucking arthropods, such as fleas, bedbugs, ticks and mosquitoes (Lighton et al., 1993; Gray and Bradley, 2003; Gray and Bradley, 2006; DeVries et al., 2013). However, the species that has been best characterized in terms of respiration dynamics and energetic cost in different situations is the kissing-bug *Rhodnius prolixus* (Bradley et al., 2003; Contreras and Bradley, 2009; Contreras and Bradley, 2010; Rolandi et al., 2014; Heinrich and Bradley, 2014; Leis et al. 2016; Paim et al. 2016). As obligated blood-feeders hemimetabolous insects, they approach their hosts mostly by walking. Hence, kissing-bugs constitute a suitable model system to evaluate the energetics of pedestrian locomotion in blood-sucking insects. *Rhodnius prolixus* is, in addition, a classical model in insect physiology and an important vector of the causative agent of a major health problem in Central and South America, Chagas disease (Lazzari, 2017).

The aim of this work is characterising energetic costs associated to walking in *R. prolixus*. Our knowledge about the evolutionary ecology of triatomines is still limited (Menu et al. 2010). In order to dig deeper on this aspect of their biology, quantitative estimates of costs and benefits associated to different activities (e.g. feeding, locomotion, egg laying, etc.) and life history traits (tolerance to starvation, reproductive strategy, dispersal, etc.) are required. This study attempts to provide information in line with this need and shed additional light on the dispersal capacities of Chagas disease vectors.

MATERIAL AND METHODS

Experimental animals and set-up

Fifth-instar nymphs of *Rhodnius prolixus* (Stål) from our laboratory colony were fed on heparinized sheep blood offered in an artificial feeder, and used for experiments 10 to 15 days after their imaginal ecdysis. Groups of insects were

maintained in a climatic chamber into 0.5 l plastic jars at $25\pm 1^{\circ}\text{C}$, $60\pm 5\%$ relative humidity and subjected to a 12h:12h light/dark cycle.

After the imaginal moult, fifteen females and males were separately weighed to the 0.1 mg, individually placed into 5 ml disposable syringes (Terumo) and kept unfed until experiments. Each insect was glued to a 1 cm long cut toothpick by the pronotum and then placed into a 30 ml respirometric chamber, made by modifying 50 ml disposable syringes, for measuring resting, walking and recovery metabolic rates. Measurements of resting metabolic rate started one minute after insects stopped moving their legs. Together with the insect, a polystyrene ball was introduced in the chamber in order to provide a walking substrate that the insect could grasp with its legs, (Rothe and Nachtigall, 1989) (Fig. 1). From several ball sizes tested, the most adequate in terms of facility for the bugs to hold, natural leg position and longer walking periods, were balls of 3.1 cm dia (341 mg). Each essay was video recorded for posterior calculation of the walking speed and dynamics.

Walking and recovery metabolism

In our study, walking was defined as the turning of a polystyrene ball by continuous movement of the insect legs as proposed by Rothe and Nachtigall (1989). Walking was stimulated by gently turning the respirometric chamber upside down until the polystyrene ball contacted the insect tarsi (Fig. 1B). A finger gently touched the external wall of the chamber in front of the starved insects, to provide a stimulus (heat) triggering walking. After 30 min trial, the respirometric chamber was gently hit to stimulate the release of the polystyrene ball by the insect, and so measuring the recovery metabolism. Afterwards, individuals were removed from the chamber, detached from the support and weighted again.

Respiratory measurements

Metabolic measurements were carried out in a room kept at a constant temperature of $25\pm 1^{\circ}\text{C}$. Flow-through respirometry following the methods already validated (Bradley et al., 2003; Contreras and Bradley, 2010; Lighton, 2008; Leis et al. 2016), was used to measure the CO_2 production and water loss. Measures were performed during resting (basal metabolism) and during and after walking using a Sable

Systems respirometric chain. *Expedata* software controlled an 8-channel multiplexer (only 2 used, one for experiments and the another as baseline) switching a water (Drierite) and CO₂ (Ascarite) scrubbed airflow of 200 ml/min (Sierra mass flow controller). The multiplexer made the airflow pass through the respirometric chambers and then conducted to a water vapour analyser (RH-300, Sable Systems, Henderson, NV, USA), after which the water was scrubbed using magnesium perchlorate. Finally, the airflow reached an infrared CO₂ analyser (CA-10, Sable Systems, Henderson, NV, USA). An electronic interphase (UI-2, Sable Systems) gathered the data from the instruments and fed them to a computer through *Expedata* for analysis.

Baseline measurements of 15 minutes were obtained using an empty chamber, both before and after each recording event, in order to determine zero CO₂-level and to correct for instrumental noise.

Metabolic calculations, data analysis and statistics

The initial 5 min of recording of the experimental and the empty chambers were excluded from the analysis to eliminate effects of the accumulated CO₂ derived from insect respiration and/or valves action. The respiratory pattern (VCO₂ min⁻¹) and the water loss (VH₂O min⁻¹) were determined during resting, during walking and during recovery. We calculated the mean rate and the mass-dependent mean rate of VCO₂ emission and water loss using the methods and equations established by Lighton (2008). To determine an energy equivalent of metabolic rate measured as the rate of CO₂ produced, we also followed the methods proposed by Lighton (2008), using the Respiratory Quotient (RQ) of 0.83 obtained by Leis et al. (2016) for resting *R. prolixus*. Walking speed was calculated from the recorded videos using the software *Tracker* (Open Source Physics). We calculated the energy cost of walking as the Gross Cost of Transport (GCOT) and the Net Cost of Transport (NCOT) after Herreid (1981), Bartholomew et al. (1988) and Schilman and Roces (2005). The movement of the polystyrene ball was fitted to a sinusoidal model by the software. A two-way ANOVA with a repeated measures factor was used to compare resting, walking and recovery and sexes. When required the variables were transformed to meet the assumptions of the model. Finally, factors were compared conducting a post-hoc Holm-Sidak pairwise multiple comparison method.

RESULTS

Body mass

In order to assess the cost of walking, first we had to characterize the dynamics associated to tethered insects walking voluntarily over a polystyrene ball.

The figure 2 depicts the body mass (mg) change after walking, as the weight decreases and the weight loss rate increases due to walking. The insects decreased their weight significantly after walking, and this regardless of the sex, i.e. female or male ($P < 0.001$; Fig. 2-A). Females were significantly heavier than males regardless the experimental conditions i.e. before or after walking ($P < 0.05$; Fig. 2-B). Detailed descriptive statistics of data is presented in the Supplementary Table A1.

Water loss

A typical respirometric run shows an augmentation of VH_2O release during walking (Fig. 3).

The analysis of \ln -transformed water loss rate (WLR; mg/h) revealed that actual, as well as \ln -transformed Mass-specific WLR (mg/h/mg) increase during walking and then decrease during recovery to a similar rate than during resting, regardless of the sex and no differences between resting and recovery were observed ($P < 0.005$; Fig. 4). Descriptive statistics is presented in the Supplementary Table A1. This increase reaches 2.4-fold for females and 2.9-fold for males.

VCO₂ emission and metabolic rate

A typical run of gas exchange is shown in Fig. 5. Prior to walking, resting bugs exhibited a cyclic pattern (CP) of CO_2 emission, showing clear and regular peaks of CO_2 release. Once an insect began to walk, gas exchange pattern became irregular and kept that way during recovery.

The computation of respirometric and energetic variables revealed that actual, as well as mas-specific metabolic rates in terms of rate of VCO_2 ($\mu\text{l/h}$) release and energetic cost, significantly increased during walking as compared to resting and recovery conditions. VCO_2 ($\mu\text{l/h}$; $P < 0.005$; Fig. 3.6-A) and Mass-specific VCO_2

($\mu\text{l/h/mg}$; $P < 0.005$; Fig. 6-B); Metabolic rate (J/h ; $P < 0.001$; Fig. 6-C); Mass-specific Metabolic rate (J/h/mg ; $P < 0.001$; Fig. 6-D). Descriptive statistics is presented in the Supplementary Table A1.

Cost of walking

Full results of the statistical analyses of metabolic variables measured in this study are presented in the Table 1.

Under our conditions, walking bugs displayed an increase in energy expenditure during walking of around 1.5-fold for females and 1.7-fold for males. The GCOT was about 421.05 J/kg/m for females and 457 J/kg/m for males. NCOT was near to 139 J/kg/m for females and 180 J/kg/m for males. Despite of the higher energy cost of walking showed by males, no significantly difference was observed associated to the sex of the individuals.

DISCUSSION

Rhodnius prolixus showed a notable weight loss after few minutes of walking. Almost 20 % of weight loss after walking during the experiments for both males and females was observed, at a rate of $1.47 \pm 0.14 \text{ mg/h}$ for females and for $0.992 \pm 0.165 \text{ mg/h}$ for males. This variation may appear as relatively high, but it should be noted that it does only refer to a short period at the beginning of the experiments, during which the insects could eliminate excrements. A similar initial weight loss has been noted by us to occur when insects were forced to walk on an arena (unpublished observation)

Our experimental bugs exhibited during resting a cyclic respiratory pattern. This pattern changes during walking, switching to an irregular gas exchange (Fig. 5), which suggests a sustained opening of spiracles. As this occurs, the rate of water loss significantly increased during walking (Fig. 3 and Fig. 4). This could explain a part of the weight loss of the insects during locomotion activity. Rolandi et al. (2014) showed a positive relationship between the WLR and the metabolic rate in *R. prolixus*. As metabolic rate and activity are also positively related, we can link the physical activity with WLR and some of the weight loss. We suppose that both water loss and weight loss stabilize during sustained walking, since as we discuss later, adults of *R. prolixus*

are potentially capable of walking considerable distances with the energy provided by a full blood meal.

Varying respiratory patterns according to behaviour

As previously described by other authors (Contreras and Bradley, 2009; Leis et al. 2016; Paim et al. 2016), *R. prolixus* displays different respiratory patterns according to their physiological condition and activity. Three different dynamics of gas exchange have been described in this species: continuous, discontinuous cyclic (DGC) and cyclic. Two of them, DGC and cyclic, have been proposed in mosquitoes to be variants of the same dynamic, being the cyclic pattern considered a manifestation of the DGC (Gray and Bradley, 2006). In the present study our time constant was 9 seconds and we used the same airflow (200 ml/min), but a different chamber size (30 ml), as employed by Contreras and Bradley (2009). Under these conditions, two different patterns clearly appeared as manifestations of gas exchange in *R. prolixus*, i.e., cyclic and continuous. Yet, in a previous article, we reported the observation of the three patterns described by other authors for this species (Leis et al., 2016).

Unfed resting bugs usually exhibit DGC (Bradley et al., 2003; Contreras and Bradley, 2009) but in our experimental conditions, insects showed a cyclic respiratory pattern before walking, maybe as a consequence of stress produced by previous manipulations or as a consequence of our time constant. Gray and Bradley (2006) suggested that insects showing a cyclic gas exchange pattern could be respiring with a discontinuous pattern. As our time constant allowed 99% of the air in the respirometric chamber to be cleared in 45 seconds, we cannot completely discard the possibility of our insects showing a DGC respiratory pattern during resting. Then, during walking a switch to continuous pattern was observed. This change was revealed in our experiments as a marked increase and dynamic change in the CO₂ emission. Water loss was predominantly continuous during both phases of our experiments, i.e. previous to and during walking, and only a high increase in the rate of water loss was observed during walking, but without change in the dynamics.

Walking energetic cost

As we have shown previously, the metabolic rate of *Rhodnius prolixus* increases during walking 1.5 times for females and 1.7 times for males as compared to the metabolic rate during resting. The energy needed to deal with this energetic demanding activity is provided by the important amount of nutrients found in blood. One hundred ml of human blood, for example, contains 0.652 g of lipids, 0.088 g of carbohydrates and 20.5 g of proteins (Lehane, 2005). If all these nutrients are metabolized, this means that the energetic value of 1 mg of human blood is approximately 3.91 J. Thus, a full blood meal (150 mg) can provide 586 J of energy and allow adults of *R. prolixus* to rest up to 43 days in females and 57 days in males (Fig. 7). These times correspond to our experimental conditions, where previous manipulation and the fact of being hanging into the chamber could induce some stress; so, longer times could be expected for completely undisturbed bugs in thigmotactic akinesis into a natural refuge. Assuming that an individual uses its available energy only for walking, a full blood meal can purvey the energy required to walk up to 15 km to females and 18 km to males, at 0.6 cm/h of average speed (Fig. 7).

Comparing the energetic cost of locomotion is not always an easy task, even when metabolic data for a single species during walking is available. The cost of locomotion can be assessed by obtaining the Gross Cost of Transport (GCOT), the Net Cost of Transport (NCOT) and/or the Minimum Cost of Transport (MCOT). GCOT is calculated by dividing the mass-dependent metabolic rate during walking by the walking speed, NCOT is determined by subtracting the mass-dependent metabolic rate during resting to the mass-dependent metabolic rate during walking and then dividing it by the walking speed. Finally, MCOT is defined as the slope of the relation between the mass-dependent metabolic rate during walking and the walking speed of each individual (Herreid et al., 1981; Schilman and Roces, 2005). The GCOT is the most direct estimation of the cost of transport and maybe the most relevant measure in an ecological approach. This measure has two main disadvantages: it is dependent on the resting metabolic rate of the animals and it drops toward minimum levels as the speed increases. The NCOT copes the resting metabolic rate issue by simply subtracting it from the walking metabolic rate, but the NCOT still drops as the speed increases. The MCOT, on the other hand, is not dependent on either the speed or the resting metabolic rates, allowing comparisons of the locomotion of organisms walking or running at

different velocities (Herreid et al., 1981; Lighton et al., 1993). In this study, the resting metabolic rates and the walking speed did not differ among the different individuals of *R. prolixus* analysed, making the calculation of GCOT and NCOT very robust (Schilman and Roces, 2005), but not the MCOT, making comparisons with other species unpractical. However, it is possible to compare the increment of the metabolic cost during locomotion related to their respective resting metabolic cost. From this perspective, the increase of metabolic cost of *R. prolixus* is not as important as some species of ants. Individuals of the ant *Camponotus sp.* shown an increment of metabolic rate of 4.3 times during level walking at 0-5 mm/s over resting rates (Lipp et al., 2005). That represents an augmentation of almost two times and 1.5 times the equivalent metabolic rate increase of females and males of *R. prolixus*, respectively. The increase associated to walking is even lower than the metabolic increase of flying over resting in terms of mass-specific rate of O₂ consumption for *Drosophila melanogaster* (Hocking, 1953; Lehmann et al., 2000; Niven and Scharlemman, 2005).

The knowledge of the cost of locomotion of blood-sucking bugs can provide valuable information about their dispersive capacity. There is just few published information of the dispersive capacity of *R. prolixus* either by walking or flight. There is a little information on field dispersive flight of another blood-sucking bug, *Triatoma infestans* but no data related to metabolic cost. *T. infestans* are capable of long flights and walking excursions (Lehane and Schofield, 1981; Abrahan et al., 2011). Assuming that flight in triatomines, as shown by other flying insects (Niven and Scharlemann, 2005), is far more expensive in terms of metabolic cost, we can argue that the dispersive capacity by walking is much higher than by flying. Our results reflect that assumption and show the first experimental data that allows to estimate how far an adult individual of *R. prolixus* can go by walking with the energy provide by a full meal on human blood, revealing an impressive dispersive capacity of almost 15 km in females and 18 km in males.

It is worth mentioning that water loss was measured under respirometric conditions, i.e., using a continuous current of dry air. Despite this fact, the loss of water during walking was only 0.5-0.7 % of the insect weight per hour, which seems relatively low for an active insect under these conditions. We can then expect that water loss would not be a limiting factor for the dispersion capacity of kissing-bugs.

Summarizing, the present work presents the first analysis of the energetic cost of walking in an haematophagous insect. We provide measured quantitative data for adults of both sexes and calculated the energetic worth of blood as energy source. This information is essential for the elaboration of realistic models of the dispersive capacity of Chagas disease vectors, as well to a comprehensive analysis of the ecophysiology, evolutionary ecology of triatomines, as well as the epidemiology of American Trypanosomiasis. Yet, a worth of caution should be given to the reader, concerning the consideration of these findings in a natural situation. The biology of kissing bugs makes particularly difficult performing metabolic measures under conditions close to an insect spontaneously walking in the wild. In the laboratory, triatomines either tend to remain immobile when undisturbed or they run when manipulated. When they are put in an arena, instead of exploring it, they usually run to reach a wall and remain in thigmotactic akinesis. On the other hand, their resting metabolic rate (Leis et al. 2016) is relatively low, as compared to more active insects, as bees or ants. To assure a reliable quantification of respiratory variables, the use of small chambers is required, as well as precise control of the experimental conditions. In order to cope with these constraints, we chose to adapt to bugs the method employed by Rothe and Nachtigal (1989) for evaluating the cost of walking in honeybees. Given that in this situation bugs are not naturally carrying their own weight in a natural way, but keeping a load with their legs, our method probably overestimates or underestimated real costs. It is worth mentioning, however, that our experimental results and estimations are in line with the dispersive potential of bugs observed in the field (Abraham et al. 2011). Despite costs could not be exact, having unravelled that water and energy reserves obtained in a single meal allow walking bugs walking along distances of several kilometres is relevant from an epidemiological point of view, even assuming them as approximative figures of such capacity.

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Author contributions

ML and CRL conceived and designed the experiments; ML carried out the respirometry experiments; ML and CRL performed statistical analyses; CRL contributed reagents/materials/analysis tools, and CRL and ML provided the conceptual frame. Both authors jointly wrote the paper.

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Data availability

<http://dx.doi.org/10.17632/x2dmzft9jc.1>

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Figures

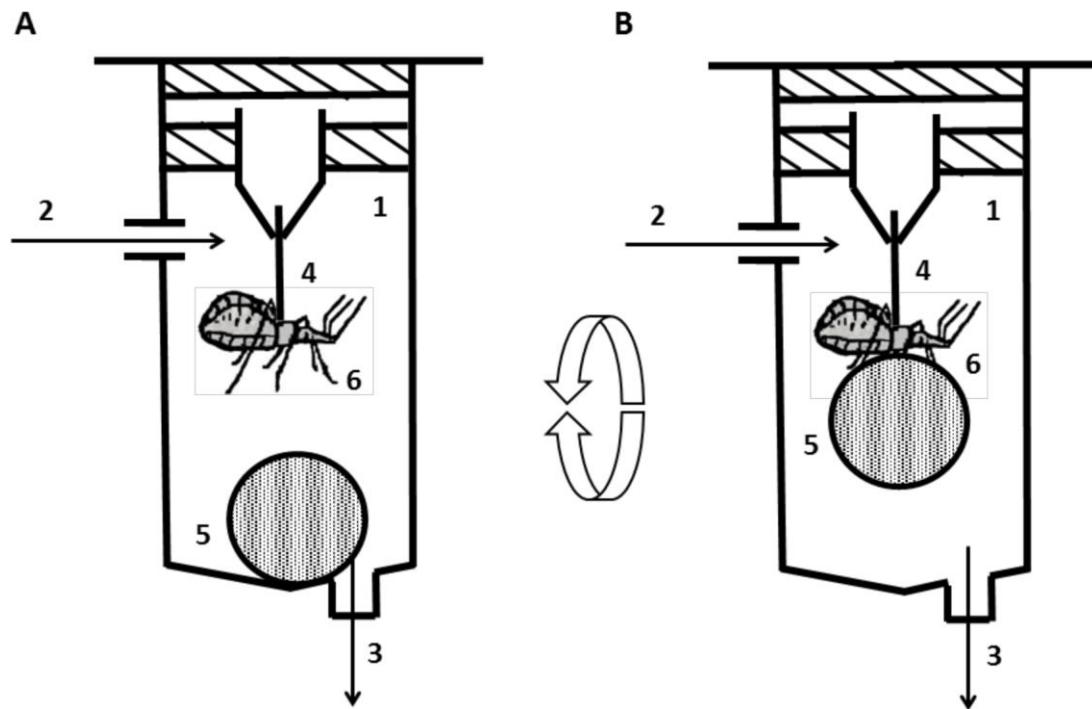


Fig. 1. Respirometric chamber for measuring CO₂ release and water loss during resting, during walking and during recovery. **A**, Resting and recovery metabolic rate setup; **B**, walking metabolic rate setup. **1**, plastic syringe; **2**, incurrent air; **3**, excurrent air; **4**, cut toothpick; **5**, polystyrene ball; **6**, Adult of *Rhodnius prolixus*.

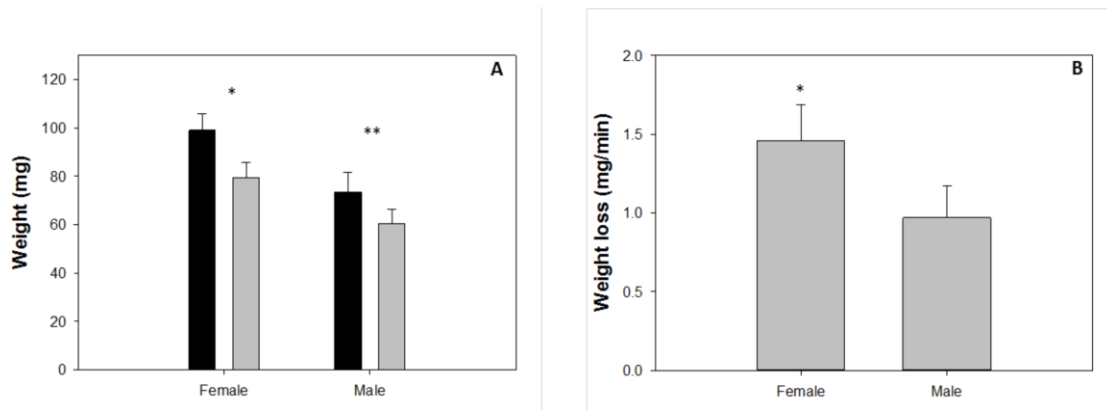


Fig. 2. Body mass of bugs before (black bars) and after (grey bars) respirometric measurements. Means + s.e.m (n= 10 for both female and male) of **A**, body mass during resting and after recovery; **B**, walking-dependent weight loss.

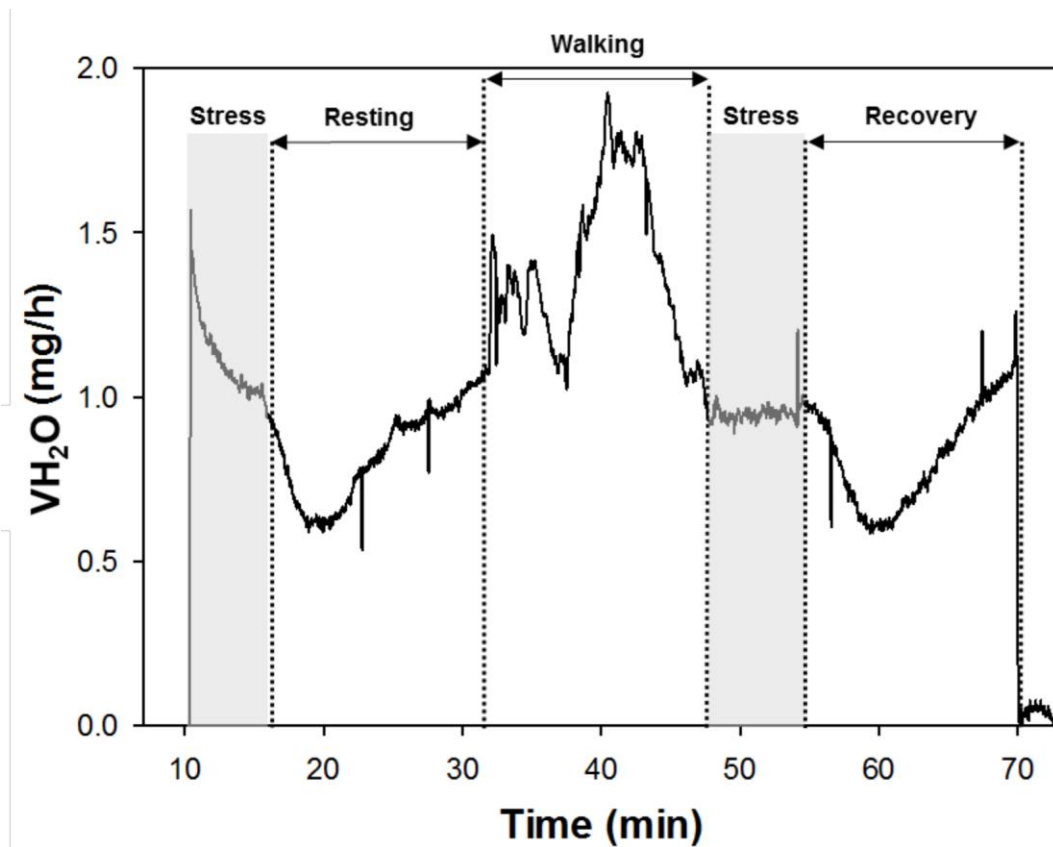


Fig. 3. Example of H₂O release for a single insect during resting (left), during walking (middle) and during recovery (right). Grey strips show periods of stress due to induced polystyrene ball grabbing or release.

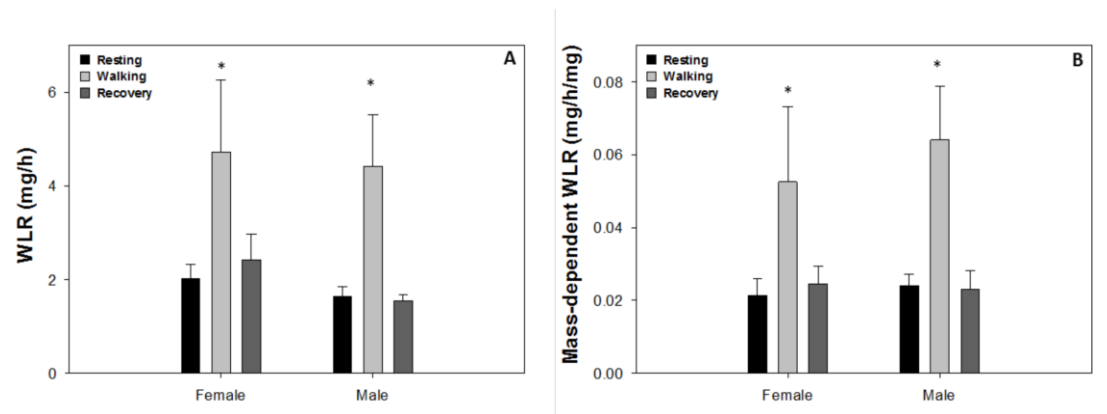


Fig. 4. Water loss associated with walking. **A**, water loss rate (WLR) during resting, during walking and during recovery and **B**, mass-specific WLR during resting, during walking and during recovery, of adults of *R. prolixus*. Means + s.e.m (n= 10 for both female and male) are represented.

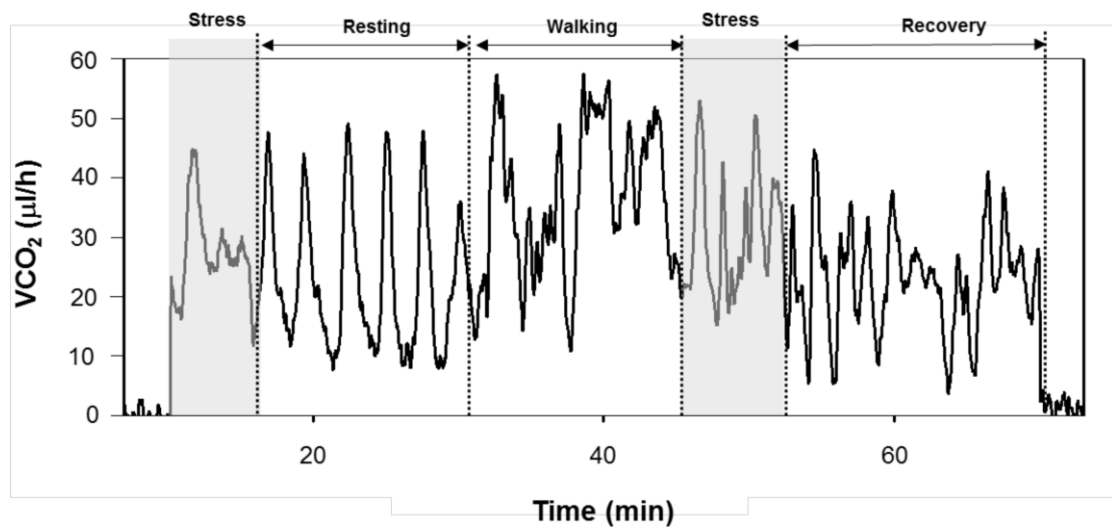


Fig. 5. Example of CO₂ release for a single insect during resting (left), during walking (middle) and during recovery (right). A change of pattern when insects started to walk was observed. Grey strips show periods of stress due to induced polystyrene ball grabbing or release.

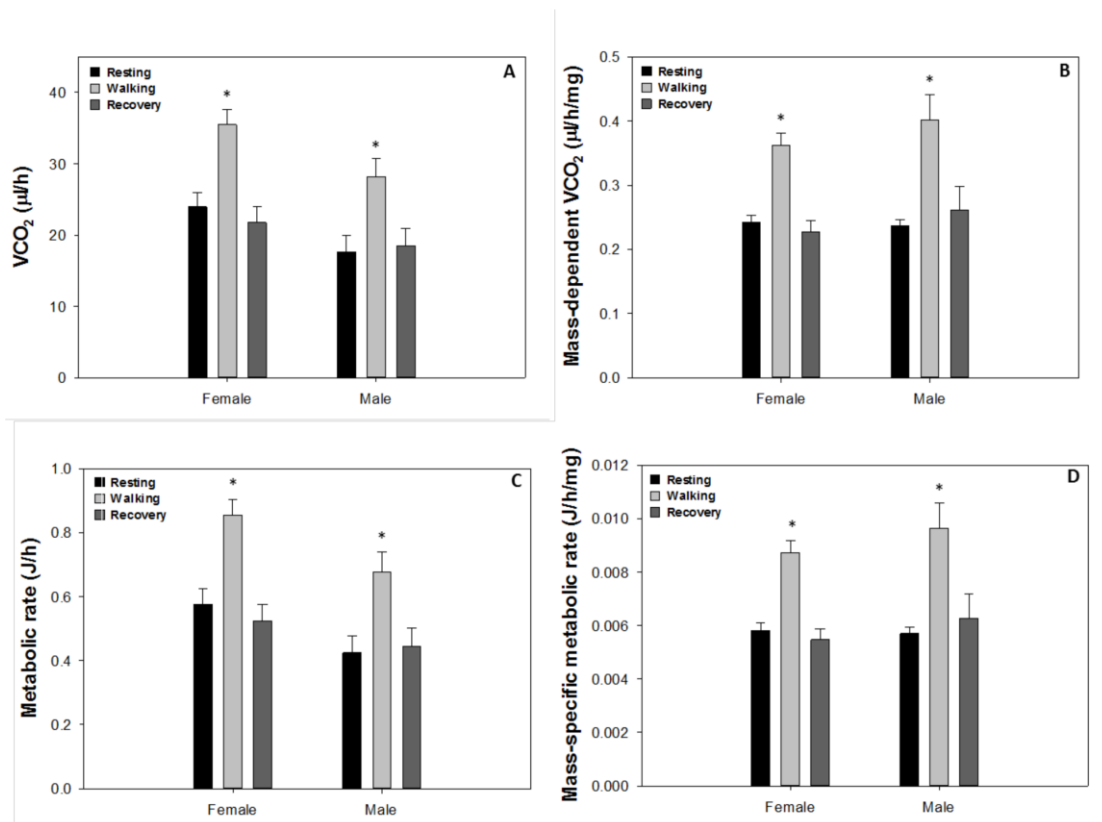


Fig. 6. Metabolic rate associated with walking. Means + s.e.m. (n=10 both female and male) of **A**, metabolic rate in terms of CO₂ release during resting, during walking and during recovery; **B**, mass-specific metabolic rate in terms of CO₂ release during resting, during walking and during recovery; **C**, metabolic rate in terms of energy cost using a RQ = 0.83 during resting, during walking and during recovery; **D**, mass-specific metabolic rate in terms of energy cost using a RQ = 0.83 during resting, during walking and during recovery.

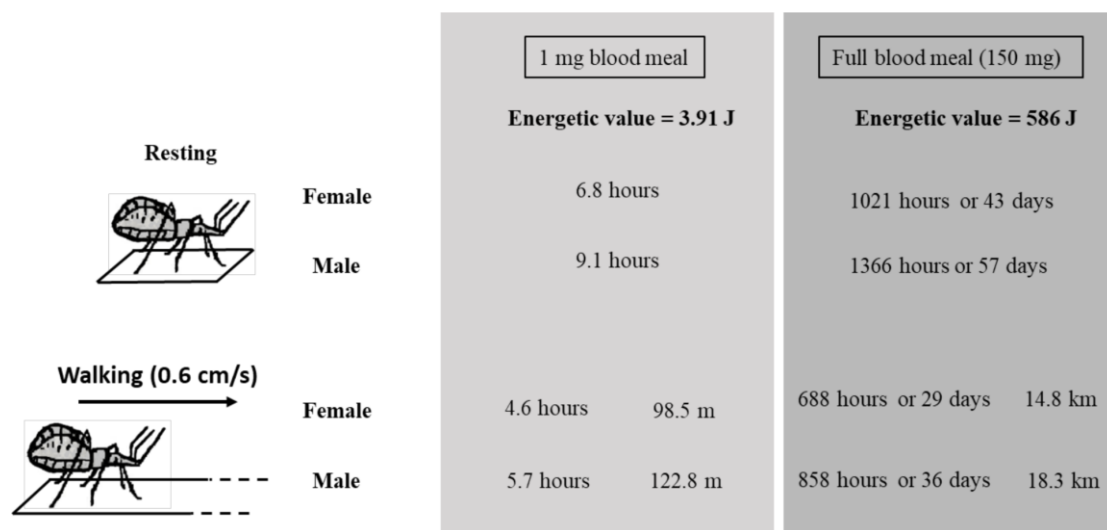


Fig. 7. Energetic value of blood. 1 mg of human blood can provide the energy for resting close to 7 hours in females and 9 hours in males of *R. prolixus*. Also, it can provide the energy for walking at 0.6 cm/s during almost 5 hours or 100 m in females and 6 hours or 123 m in males.

Table 1. Results of statistical analyses. Detailed descriptive statistics is presented in the Supplementary Table A1.

Figure	Variable	Condition / Sex	Statistic	P-value
2-A	Weight (mg)	Females	t= 9.508	<0.005
		Males	t= 5.589	<0.001
2-B	Weight loss (mg/min)	Before	t= 2.424	<0.05
		After	t= 2.197	
4. A-B	In-transformed WLR (mg/h) and In-transformed Mass-specific WLR (mg/h/mg)	All	F _{2,40} = 40.54	<0.001
		Walking vs. Resting	t= 7.970	
		Walking vs. Recovery	t= 7.530	
		Walking vs. Resting within Female	t= 4.734	<0.005
		Walking vs. Recovery within Female	t= 3.825	
		Walking vs. Resting within Male	t= 6.537	<0.001
		Walking vs. Recovery within Male	t= 6.920	
6. A-B	VCO ₂ (μl/h) and Mass-specific VCO ₂ (μl/h/mg)	All	F _{2,40} = 32.179	<0.001
		Walking vs. Resting	t= 7.077	
		Walking vs. Recovery	t= 6.737	
		Walking vs. Resting within Female	t= 4.250	
		Walking vs. Recovery within Female	t= 4.626	
		Walking vs. Resting within Male	t= 5.759	
		Walking vs. Recovery within Male	t= 4.914	
6-C	Metabolic rate (J/h)	All	F _{2,40} = 43.591	<0.001
		Walking vs. Resting	t= 8.029	
		Walking vs. Recovery	t= 8.062	
		Walking vs. Resting within Female	t= 5.937	
		Walking vs. Recovery within Female	t= 6.369	
		Walking vs. Resting within Male	t= 5.418	
		Walking vs. Recovery within Male	t= 4.998	
6-D	Mass-specific Metabolic rate (J/h/mg)	All	F _{2,40} = 32.179	<0.001
		Walking vs. Resting	t= 7.077	
		Walking vs. Recovery	t= 6.737	
		Walking vs. Resting within Female	t= 4.250	
		Walking vs. Recovery within Female	t= 4.626	
		Walking vs. Resting within Male	t= 5.759	
		Walking vs. Recovery within Male	t= 4.914	

Table S1. Descriptive statistics (means \pm s.e.m) of weight, speed, metabolic values and water loss in both sexes. Results of statistical analyses can be found in the Table 1.

	Female	Male
Temperature ($^{\circ}$ C)	25	25
n	10	10
Weight before walking (mg)	99.68 \pm 5.17	72.88 \pm 5.6
Weight loss (mg)	19.78 \pm 1.49	13.19 \pm 1.94
Weight loss rate (mg/min)	1.47 \pm 0.14	0.992 \pm 0.165
RMR (μ l CO ₂ /h)	23.88 \pm 1.54	17.83 \pm 1.82
Mass-dependent RMR (μ l CO ₂ /h/mg)	0.242 \pm 0.009	0.237 \pm 0.007
RMR (J/h)	0.574 \pm 0.037	0.429 \pm 0.043
Mass-dependent RMR (J/h/mg)	0.00583 \pm 0.0002	0.0057 \pm 0.0002
Speed (cm/s)	0.596 \pm 0.041	0.594 \pm 0.013
Walking metabolic rate (μ l CO ₂ /h)	35.42 \pm 1.53	28.40 \pm 1.87
Mass-dependent Walking metabolic rate (μ l CO ₂ /h/mg)	0.364 \pm 0.013	0.401 \pm 0.03
Walking metabolic rate (J/h)	0.852 \pm 0.037	0.683 \pm 0.045
Mass-dependent Walking metabolic rate (J/h/mg)	0.00877 \pm 0.0003	0.00965 \pm 0.0007
GCOT (J/Kg/m)	421.05 \pm 34.31	457.48 \pm 28.28
NCOT (J/Kg/m)	138.7 \pm 19.75	179.52 \pm 27.7
Recovery metabolic rate (μ l CO ₂ /h)	21.6 \pm 1.37	18.91 \pm 1.68
Mass-dependent Recovery metabolic rate (μ l CO ₂ /h/mg)	0.228 \pm 0.01	0.266 \pm 0.028
Recovery metabolic rate (J/h)	0.519 \pm 0.033	0.782 \pm 0.18
Mass-dependent Recovery metabolic rate (J/h/mg)	0.00549 \pm 0.0002	0.0117 \pm 0.003
Resting WLR (mg/h)	2.003 \pm 0.211	1.65 \pm 0.14
Resting mass-dependent WLR (mg/h/mg)	0.0215 \pm 0.003	0.0239 \pm 0.025
Walking WLR (mg/h)	4.8 \pm 1.1	4.5 \pm 0.82
Walking Mass-dependent WLR (mg/h/mg)	0.0538 \pm 0.015	0.065 \pm 0.011
Recovery WLR (mg/h)	2.426 \pm 0.32	1.55 \pm 0.11
Recovery Mass-dependent WLR (mg/h/mg)	0.0245 \pm 0.003	0.0234 \pm 0.004