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RESEARCH ARTICLE

Metabolism and water loss rate of the haematophagous insect *Rhodnius prolixus*: effect of starvation and temperature

Carmen Rolandi[1,2], Mónica S. Iglesias[1] and Pablo E. Schilman[1,2,*]

ABSTRACT

Haematophagous insects suffer big changes in water needs under different levels of starvation. *Rhodnius prolixus* is the most important haematophagous vector of Chagas disease in the north of South America and a model organism in insect physiology. Although there have been some studies on patterns of gas exchange and metabolic rates, there is little information regarding water loss in *R. prolixus*. We investigated whether there is any modulation of water loss and metabolic rate under different requirements for saving water. We measured simultaneously CO_2 production, water emission and activity in individual insects in real time by open-flow respirometry at different temperatures (15, 25 and 35°C) and post-feeding days (0, 5, 13 and 29). We found: (1) a clear drop in metabolic rate between 5 and 13 days after feeding that cannot be explained by activity and (2) a decrease in water loss rate with increasing starvation level, by a decrease in cuticular water loss during the first 5 days after feeding and a drop in the respiratory component thereafter. We calculated the surface area of the insects and estimated cuticular permeability. In addition, we analysed the pattern of gas exchange; the change from a cyclic to a continuous pattern was affected by temperature and activity, but it was not affected by the level of starvation. Modulation of metabolic and water loss rates with temperature and starvation could help *R. prolixus* to be more flexible in tolerating different periods of starvation, which is adaptive in a changing environment with the uncertainty of finding a suitable host.

KEY WORDS: Flow-through respirometry, Respiratory water loss, Cuticular permeability, CO_2 emission rate

INTRODUCTION

Desiccation resistance is vital for survival and colonization of terrestrial habitats. In insects in particular, there must be a fine and efficient control of water loss because of their high surface area to volume ratio. Insects lose water through various pathways: transpiration through the cuticle, evaporation along open spiracles through the tracheal system, and excretion (Edney, 1977; Hadley, 1994). The contribution of each of these pathways to overall water loss is variable but cuticular water loss (CWL) generally accounts for a high proportion of the total water loss (Gibbs and Johnson, 2004; Hadley, 1994). The contribution of respiratory water loss (RWL) to dehydration has been analysed mostly in insects showing discontinuous gas exchange (DGE) (e.g. Chown and Davis, 2003; Lighton, 1992; Quinlan and Lighton, 1999). There are two

[1]Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, C1428EHA, Buenos Aires, Argentina. [2]Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA), CONICET-UBA, C1428EHA, Buenos Aires, Argentina.

*Author for correspondence (schilman@bg.fcen.uba.ar)

techniques that enable CWL and RWL to be distinguished in insects with continuous gas exchange: the regression method (Gibbs and Johnson, 2004) and the hyperoxic switch method (Lighton et al., 2004). Using these techniques, it was observed that spiracular control under continuous gas exchange can modulate RWL as effectively as DGE (Schilman et al., 2005; Gray and Chown, 2008).

Haematophagous insects that do not drink free water show big changes in water balance under different levels of starvation (Benoit and Denlinger, 2010). Immediately after feeding they have to release large amounts of water and then, depending on the species, they can spend days, months or even years without feeding (Wigglesworth, 1972). During this time, the insects are under huge pressure to keep water. The haematophagous bug *Rhodnius prolixus* Stål 1859 (Hemiptera: Reduviidae) is an important vector of Chagas disease in northern South America and Central America, and it remains a classical model in insect physiology. It is distributed over Venezuela and Colombia, where it mainly inhabits wild environments such as palm trees, while in Central America (especially in Guatemala, Honduras and El Salvador) it has adapted to domestic environments (Schofield, 1994). Abiotic factors such as temperature and water availability are important for the distribution and abundance of insect species, which frequently show adaptations to their environments (Chown and Nicolson, 2004). *Rhodnius prolixus* are mostly associated with xeric regions such as dry savannah areas, making them an interesting model to assess the control of water loss.

Metabolic rate was studied in *R. prolixus* males in relation to the gas exchange pattern and the effect of temperature (Contreras and Bradley, 2009; Contreras and Bradley, 2010). Although it is considered that the pattern of DGE could represent an advantage for hygric efficiency (White et al., 2007; Terblanche et al., 2010), all of the previous work has focused on patterns of gas exchange and metabolic rate, and there is little information regarding water loss through the cuticle and spiracles in *R. prolixus*. The aim of the present study was to investigate the modulation of water loss rate (WLR) and metabolic rate under different requirements for water and nutrient conservation. To do this, we simultaneously measured CO_2 production, water vapour emission and activity in individual insects in real time by open-flow respirometry at different feedings states and temperatures. In addition, we estimated the surface area of the insects, distinguished between CWL and RWL by the regression method, and calculated cuticular permeability.

RESULTS
Metabolic rate

A typical run is shown in Fig. 1. Gas exchange patterns changed from continuous to cyclic in the presence and absence of activity, and also varied across treatments as reflected by the variability coefficient (VC) of the CO_2 emission rate ($\dot{V}_{CO_2}$) (Table 1). Repeated measures ANOVA of the ln-transformed VC revealed that temperature had a significant effect on the variability of metabolic rate within each recording ($F_{2,32}=53.7$, $P<0.0001$). The interaction
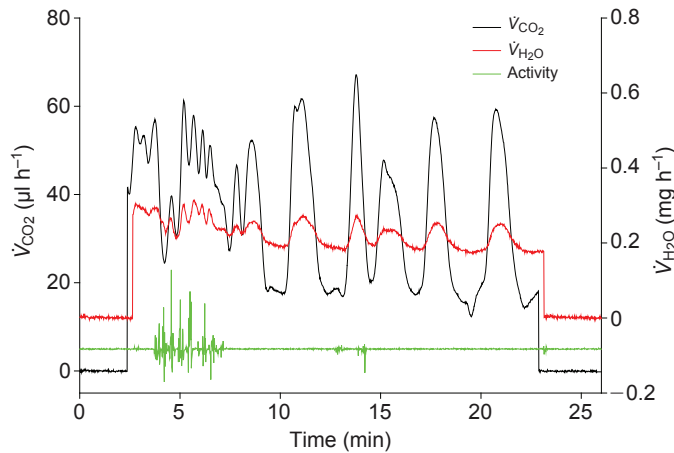
Fig. 1. Typical recording of CO_2 production and water release from *Rhodnius prolixus*. The extreme left and right portions of the recording correspond to baseline values, which represent measurements made on an empty chamber. Recordings of the insect (mass 117.7 mg) were made at 25°C on the 5th day post-feeding. Traces represent CO_2 production ($\dot{V}_{CO_2}$) and H_2O release ($\dot{V}_{H_2O}$), together with activity (measured in arbitrary units).

between factors was not significant ($F_{6,96}$=1.67, P=0.14). Insects measured at 35°C showed a smaller VC, indicating a higher degree of continuity of gas exchange, whereas the VC for insects measured at 15 and 25°C was statistically homogeneous. Throughout the days after feeding, VC remained constant as the proportion of runs where cyclic gas exchange was observed (Table 1). The probability of cyclic gas exchange presence was consistent with the VC results (supplementary material Fig. S1). We also calculated Pearson's coefficients of correlation between the VC of $\dot{V}_{CO_2}$ and total WLR (Fig. 2). Because insects were measured repeatedly over the post-feeding days, and in order to maintain independence between samples, for this analysis we used the mean variables of each insect tested. Pearson product-moment correlation indicated a significant negative association between the VC of the $\dot{V}_{CO_2}$ trace and total water loss at 25°C (r=–0.69, P=0.013) and a marginal association at 35°C (r=–0.58, P=0.05), while at 15°C there was no association between these variables (r=–0.46, P=0.15) (Fig. 2).

Body mass did not differ across temperature ($F_{2,32}$=0.17, P=0.89, repeated measures ANOVA; Table 1). Overall, metabolic rate increased with temperature and decreased with nutritional state (Fig. 3), and the interaction of these factors was significant ($F_{6,96}$=4.81, P=2×10^{-4}). Metabolic rate remained constant during the first two nutritional intervals tested, with a tendency to decrease between 5 and 29 days after feeding. At 15°C, a significant decrease was registered on the 29th day after feeding, while at 35°C, a significant decrease occurred on the 13th day after feeding, and then metabolic rate remained constant through to the 29th day. For measurements made at 25°C, there were no significant differences between the rates at 0 and 5 days after feeding, and at 0 and 13 days after feeding; however, the metabolic rate on the 29th day differed significantly from all the other starvation levels assessed (Fig. 3, Table 1).

As a way to account for specific dynamic action (SDA), we calculated the quotient between metabolic rate measured during the hours following feeding and 29 days after feeding. The increase in metabolic rate as a consequence of feeding was 1.96-fold at 15°C, 2.26-fold at 25°C and 1.86-fold at 35°C. The effect of temperature was not significant ($F_{2,32}$=2.41, P=0.11, one-way ANOVA). Overall, the mean increase in $\dot{V}_{CO_2}$ was 2.03-fold. The four nutritional states

Table 1. Summary means of body mass, activity and metabolic rate

	Day 0			Day 5			Day 13			Day 29		
Temperature (°C)	15	25	35	15	25	35	15	25	35	15	25	35
N	11	12	12	11	12	12	11	12	12	11	12	12
Mass (mg)	111.3±4.2	114.8±4.7	116.1±4.1	89.2±3.8	92.6±4.3	91.9±3.9	74.7±4.0	74.0±3.3	75.5±4.2	51.6±3.4	52.3±3.1	50.8±2.9
Activity (a.u.)	23.23±6.07	53.44±20.97	83.99±16.64	20.80±5.07	50.19±12.47	48.62±10.65	31.35±8.88	70.91±15.90	58.40±19.73	13.06±1.17	31.86±8.32	160.76±28.15
$\dot{V}_{CO_2}$ (µl h^{-1})	9.08±0.73	19.74±2.27	45.08±2.90	10.98±0.98	24.17±1.32	45.61±2.65	7.64±0.70	15.76±1.16	26.84±1.77	4.93±0.56	8.84±0.81	26.27±1.97
VC	0.70±0.07	0.60±0.05^A	0.21±0.01^B	0.67±0.04^A	0.59±0.05^A	0.32±0.03^B	0.75±0.06^A	0.63±0.07^A	0.34±0.03^B	0.75±0.09^A	0.77±0.12^A	0.28±0.02^B
Q_{10}	2.14			2.12			1.89			2.10		

Data (means and s.e.) are reported for days 0, 5, 13 and 29 post-feeding. Metabolic rate is shown as the rate of CO_2 production, $\dot{V}_{CO_2}$. N, sample size; VC, variability coefficient of the CO_2 emission rate (for a detailed explanation, see Materials and methods); a.u., arbitrary units.
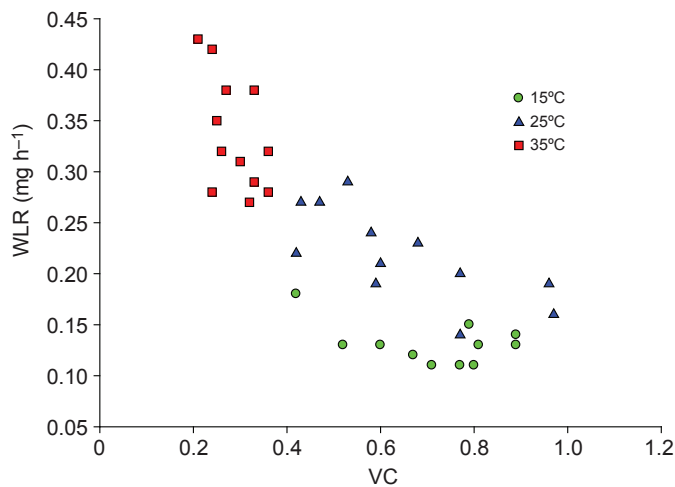Values with the same superscript letters are statistically homogeneous between temperature treatments.

Fig. 2. Correlation between water loss rate (WLR) and variability coefficient (VC) of $\dot{V}_{CO_2}$. Variability of the CO_2 trace was calculated as the s.d. mean ratio. Each point is an average of four measurements of the same insect.

did not differ in temperature sensitivity of the metabolic rate, or slope (ANCOVA of log-transformed metabolic rate versus temperature) ($F_{3,132}=2.51$, $P=0.06$). The lines possess a common slope of 0.033 ± 0.001, which corresponds to a Q_{10} of $10^{10\times0.033}$ or 2.13 (Lighton, 2008). Intercepts did differ significantly ($F_{1,135}=604.72$, $P<1\times10^{-5}$).

As well as metabolic rate, activity values [expressed as the absolute difference sum (ADS) of activity signal measured in volts; for a detailed explanation of the activity measurement, see Materials and methods, 'Respirometry' and 'Analyses and statistics'] showed a significant interaction between temperature and starvation ($F_{6,96}=3.55$, $P=3.2\times10^{-3}$). This relationship was mainly due to the increased movement of insects measured on post-feeding day 29 at 35°C (Table 1; supplementary material Fig. S2). Activity was lower in insects measured at 15 and 25°C than at 35°C, and it was not
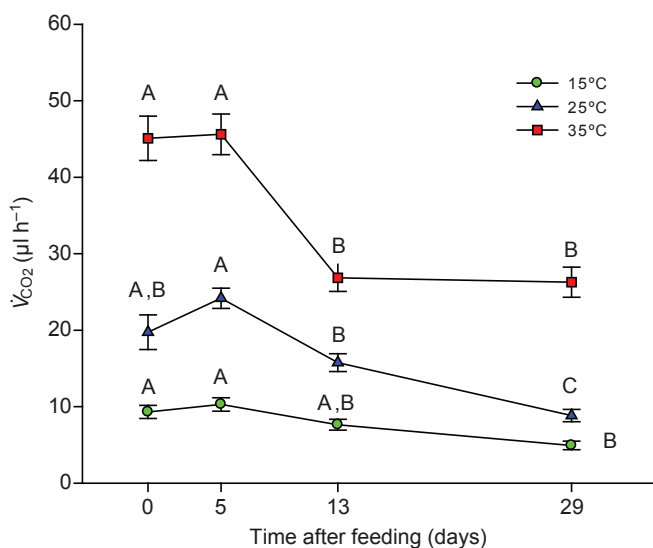
affected by the starvation level (Tukey *a posteriori* test; supplementary material Fig. S2).

Water loss measurements
Repeated measures ANOVA of square root-transformed WLR revealed significant differences depending on the feeding state ($F_{1,32}=40.39$, $P<1\times10^{-4}$) and temperature ($F_{2,32}=74.24$, $P<1\times10^{-4}$). The interaction between the two factors was not significant ($F_{6,96}=1.55$, $P=0.23$). WLR showed a significant decrease between 0 and 5 days post-feeding, and continued to decrease less steeply as starvation increased (Fig. 4). We used the regression method in order to assess the cuticular and respiratory components of WLR (Gibbs and Johnson, 2004). All regressions showed significant positives slopes (supplementary material Table S1). Four regressions were excluded from analysis because their R^2 values were lower than 0.1; the remaining R^2 values varied between 0.21 and 0.96 (supplementary material Table S1). A similar change to WLR was observed in the CWL rate profile, with a significant positive effect of temperature ($F_{2,28}=38.84$, $P<1\times10^{-4}$) and a negative effect of feeding state ($F_{3,84}=23.89$, $P<1\times10^{-4}$). There was a significant decline of water loss through the cuticle between the hours following feeding (and diuresis) and the 5th post-feeding day, without further changes throughout the subsequent days (Table 2). However, for RWL rate, there was a significant interaction between temperature and post-feeding day ($F_{6,86}=3.38$, $P=0.005$; Table 2); RWL reached its lowest value on days 13 and 29 for insects measured at 35 and 25°C, respectively, while it remained constant throughout the post-feeding days for insects measured at 15°C.

Using Eqn 4 (see Materials and methods), which estimates wingless surface area, together with WLR and CWL rate, we calculated gross cuticular permeability (GCP) and cuticular permeability (CP). There was a negative effect of temperature (GCP: $F_{2,32}=5.72$, $P=7.5\times10^{-3}$; CP: $F_{2,28}=14.43$, $P<1\times10^{-4}$) and nutritional level (GCP: $F_{3,96}=25.27$, $P<1\times10^{-4}$; CP: $F_{3,84}=13.83$, $P<1\times10^{-4}$) on GCP and CP. The interaction between the two factors was not significant for any of the variables tested. Estimates of CP of insects measured at 35°C were significantly lower than those for insects at 15 and 25°C, and GCP values were significantly lower at 35°C than at 15°C. The effect of nutritional level followed the same profile as CWL rate for GCP and CP (Table 2).
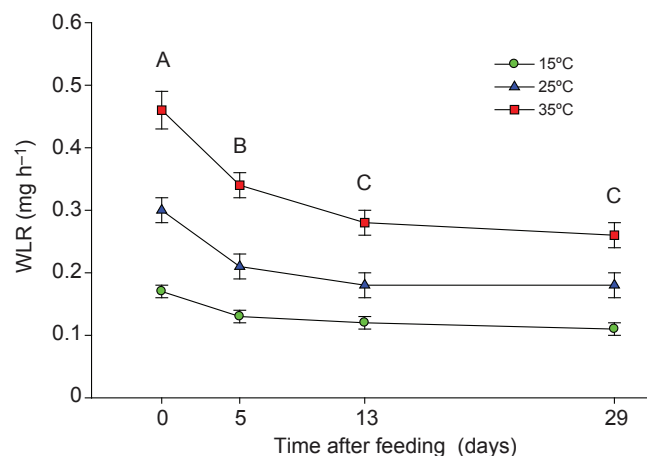

Fig. 3. Effects of temperature and feeding state on metabolic rate. Measurements throughout the post-feeding days for the same temperature were performed on the same individuals. Different letters show significant differences of metabolic rate between post-feeding days for each treatment (Tukey test; $P<0.01$).

Fig. 4. Effects of temperature and feeding state on WLR. WLR is shown for different temperature treatments at 0, 5, 13 and 29 days post-feeding. Different letters show significant differences between WLR for the three treatments ($P<0.01$).

Table 2. Summary means of water loss rate, together with estimates of gross cuticular permeability and corrected cuticular permeability for different feeding states and temperatures

	Day 0			Day 5			Day 13			Day 29		
Temperature (°C)	15	25	35	15	25	35	15	25	35	15	25	35
N	11	12	12	11	12	12	11	12	12	11	12	12
WLR (mg h⁻¹)	0.170±0.013[A,a]	0.300±0.024[B,a]	0.461±0.025[C,a]	0.130±0.011[A,b]	0.211±0.021[B,b]	0.341±0.024[C,b]	0.120±0.009[A,b,c]	0.180±0.018[B,b,c]	0.282±0.021[C,b,c]	0.106±0.014[A,c]	0.177±0.019[B,c]	0.264±0.016[C,c]
CWLR (mg h⁻¹)	0.140±0.010[A,a]	0.224±0.025[B,a]	0.278±0.021[C,a]	0.101±0.010[A,b]	0.146±0.020[B,b]	0.221±0.012[C,b]	0.096±0.007[B,b]	0.124±0.009[C,b]	0.202±0.009[C,b]	0.082±0.016[A,b]	0.130±0.012[B,b]	0.169±0.011[C,b]
RWLR (mg h⁻¹)	0.020±0.002[a]	0.056±0.010[a]	0.167±0.022[a]	0.022±0.003[a]	0.053±0.008[a]	0.115±0.019[a,b]	0.017±0.001[a]	0.039±0.006[a,b]	0.073±0.018[a,b]	0.017±0.005[a]	0.020±0.005[b]	0.088±0.013[b]
Surface area (cm²)	2.57±0.02	2.59±0.03	2.61±0.03	2.44±0.02	2.45±0.02	2.46±0.03	2.36±0.02	2.36±0.02	2.38±0.02	2.28±0.01	2.28±0.01	2.28±0.01
Surface area* (cm²)	1.87±0.05	1.91±0.05	1.93±0.04	1.61±0.01	1.65±0.01	1.65±0.01	1.43±0.05	1.42±0.04	1.44±0.05	1.12±0.05	1.13±0.05	1.11±0.04
GCP (µg h⁻¹ cm⁻² Torr⁻¹)	5.11±0.38[A,a]	4.89±0.37[A,B,a]	4.19±0.22[B,a]	4.14±0.34[A,b]	3.65±0.36[A,B,b]	3.29±0.22[B,b]	3.93±0.31[A,b]	3.24±0.32[A,B,b]	2.81±0.20[B,b]	3.59±0.45[A,b]	3.29±0.34[A,B,b]	2.75±0.17[B,b]
CP (µg h⁻¹ cm⁻² Torr⁻¹)	4.11±0.38[A,a]	3.64±0.37[A,a]	2.51±0.17[B,a]	3.20±0.33[A,b]	2.53±0.35[A,b]	2.13±0.10[B,b]	3.12±0.28[A,b]	2.24±0.31[A,b]	2.01±0.08[B,b]	2.76±0.53[A,b]	2.43±0.21[A,b]	1.76±0.11[B,b]

Data (means and s.e.) are reported for days 0, 5, 13 and 29 post-feeding. WLR, water loss rate; CWLR, cuticular water loss rate; RWLR, respiratory water loss rate; GCP, gross cuticular permeability; CP, corrected cuticular permeability.

Values with the same superscript letters are statistically homogeneous between temperature treatments; uppercase letters represent comparisons between temperatures and lowercase letters represent comparisons between post-feeding days.

WLR, CWLR and RWLR were analysed by repeated measures ANOVA followed by a posteriori Tukey tests. GCP and CP were analysed by GLS followed by a posteriori LSD Fisher test.

*Calculated using Meeh's formula ($S=k×W^{0.667}$), where k=8.1 (species-specific constant) (Wigglesworth, 1945) and W is mass (in g).

DISCUSSION
Metabolic rate
Although the present study was not specifically designed to test the SDA response, i.e. the metabolic response that accompanies meal ingestion, digestion, absorption and assimilation (Secor, 2009), when we focused on the effect of digestion on the metabolic rate of *R. prolixus*, we registered a postprandial metabolic scope of 2. Previous work on 5th instar nymphs of *R. prolixus* showed metabolic scope values of almost 8 and 14 (Bradley et al., 2003; Heinrich and Bradley, 2014). These higher values previously found could be explained by the larger amount of blood ingested by nymphs compared with adult males, together with the effects of other physiological processes such as development and moulting, which occur following feeding. In addition, we chose to work only with males in order to remove the effect of oogenesis on metabolic rate (Davey, 1993). Metabolic rate did not decrease immediately, but remained high for a time, decreasing between the 5th and 13th day after feeding. For crickets, during the first days of food deprivation, carbohydrate reserves were consumed, and from then on, metabolism was powered by lipids (Sinclair et al., 2011). It must be noted that we did not take into account the possibility of a change in fuel occurring as starvation increased, as undigested blood is stored in the anterior midgut and is transported into the posterior midgut for digestion and absorption as energy is required. The metabolic demands of the insects tested are mainly movement and digestion. The higher metabolic rates between days 0 and 5 post-feeding are not explained by different activity levels, as the activity ADS levels did not vary throughout the post-feeding days (except for the high values registered at 35°C on the 29th day; see supplementary material Fig. S2). In *R. prolixus*, the time for consumption of a blood meal has been estimated to be ~26 days in insects reared at 24°C and ~13 days in insects reared at 32°C (Schilman and Lazzari, 2004). The significant drop in the $\dot{V}_{CO_2}$ observed between days 5 and 13 after feeding due to cessation of the SDA effect could be used as an indicator of nutritional status, showing the transition from fed to fasted insect.

In addition to the increase of metabolic rate with digestion, there was an increase with temperature. The sensitivity of the metabolic rate to temperature (Q_{10}) was about 2 (Table 1), similar to most of the tracheate arthropods studied so far. This value is slightly lower than the Q_{10} of 2.48 found in the giant red velvet mite (Lighton and Duncan, 1995), which was used by Bradley et al. (Bradley et al., 2003) for temperature corrections of the metabolic rate in *R. prolixus*. Temperature affects not only the rate of CO_2 production but also the pattern of release (Basson and Terblanche, 2011). We found, consistent with Contreras and Bradley (Contreras and Bradley, 2010), that higher temperatures (significant differences at 35°C) and activity negatively affected the occurrence of cyclic patterns. In Contreras and Bradley's study, $\dot{V}_{CO_2}$ was measured on *R. prolixus* males fasted for 1 week at the same temperatures that we used (Contreras and Bradley, 2010). They observed DGE at 15°C, a cyclic pattern at 25°C and continuous gas exchange at 35°C. In contrast, we registered gas exchange patterns that ranged from cyclic to continuous. We did not observe DGE *sensu stricto* in any insect, perhaps due to the low flow rate used (Gray and Bradley, 2006), although Terblanche and Chown (Terblanche and Chown, 2010) showed that flow rates are only likely to be a problem at extremely low values in very small insects (i.e. at the lower operational limit of the gas analyser). However, the relationship between nutritional state and gas exchange pattern was unclear, suggesting a doubtful relationship between water needs and cyclicity. A similar lack of association between water needs and cyclic gas exchange was

recently found in the Table Mountain cockroach, *Aptera fusca* (Groenewald et al., 2013).

WLR and cuticular permeability

Like metabolic rate, WLR was lower at higher levels of starvation. Using the regression method (Gibbs and Johnson, 2004), we were able to analyse the respiratory and cuticular contribution of total water loss. The general decrease in the rate of water loss as blood reserves diminished can be explained by a decline in the cuticular water loss during the first 5 days after feeding and by a drop in the respiratory component thereafter (Fig. 4, Table 2).

After carefully measuring every part of the body of *R. prolixus*, we estimated the surface area based on body mass and length of the 3rd tibia. As all vectors of Chagas disease have a similar body shape, this model will be useful for any of the triatomine species. In addition, because they are hemimetabolous insects, nymphs and adults are also similar in body shape therefore the model could be applied to all species and larval stages. Based on WLR and estimates of the surface area, we calculated cuticular permeability, which was between 1.76 and 4.11 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$ (where 1 Torr≈133 Pa) depending on the starvation level and temperature, in agreement with values of cuticular permeability of fed nymphs of the 5th instar of *R. prolixus*; the latter was measured as the percentage of weight lost in 24 h at 30°C (Wigglesworth, 1945). Consequently, we modified Wigglesworth's data to compare with our results; cuticular permeability was 1.68 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$. This result is very similar to our lowest value measured, although a different developmental stage was used and the surface area was estimated using Meeh's formulae [our surface area measurements were between 26% and 51% (average 37%) higher than Meeh's formulae estimates]. Moreover, WLR was measured using a gravimetric technique that has been shown to yield lower values than water loss measurements made with open flow respirometry (Schilman et al., 2007). Compared with that of other species, cuticular permeability of *R. prolixus* was low, which is a characteristic of arthropods adapted to xeric environments [see table 6 from Edney (Edney, 1977) and table 3.1 from Hadley (Hadley, 1994)]. However, it was higher than the lowest cuticular permeability measured so far in the tenebrionid beetle *Onymacris plana* from the Namib Desert [0.75 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$ (Nicolson et al., 1984)] and the lowest measured by open flow respirometry in another tenebrionid beetle, *Eleodes obscura*, with 0.9 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$ (Schilman et al., 2008).

The steep and significant decrease in the cuticular water loss and cuticular permeability observed between the hours following feeding and the 5th day was probably due to a change in the surface area exposed due to unfolding of inter-segmental membrane and the separation of the wings from the abdomen as a consequence of engorgement. The latter was not taken into account in the estimates of cuticular permeability, though the unfolding of the inter-segmental membrane was accounted for. However, each local region of the cuticle has different levels of sclerotization, with different cuticular permeabilities existing between them (Andersen, 2010). The inter-segmental membrane is less sclerotized with a higher permeability per unit of surface area than the rest of the body (Andersen, 2010). However, we cannot discard the existence of another factor downregulating cuticular permeability as the time after feeding increases. In another blood-feeding arthropod, the lone star tick, a higher cuticular permeability was observed in feeding ticks, and after host drop-off, a decrease in water loss together with a 3-fold boost in the surface wax deposition was measured (Yoder et al., 1997). This might favour water loss through the cuticle as an inexpensive way to concentrate blood. Our insect model has a

different life cycle and feeding behaviour; nonetheless, the hypothesis that modulation of cuticular WLR and cuticular permeability after feeding might occur by deposition of extra surface wax or a change of the hydrocarbon chain composition on the days following feeding remains to be tested. A change in cuticular composition is likely as in a closely related triatomine, *Triatoma infestans*, a 3-fold increase of epicuticular lipids was measured between young and old adult males (Juárez et al., 1984).

An effect on cuticular permeability of feeding state was also observed in another haematophagous arthropod, the rabbit tick *Haemaphysalis leporispalustris*, where engorged nymphs showed a decreasing cuticular permeability with increasing starvation during the first 2 weeks after feeding (Davis, 1974). At high temperatures, changes in cuticular permeability have been observed as a result of melting of cuticular waxes, being over 50°C for *R. prolixus* nymphs (Wigglesworth, 1945). We therefore expected cuticular permeability to remain constant in the range of temperatures tested, i.e. between 15 and 35°C. We found, however, a significantly lower cuticular permeability of *R. prolixus* at 35°C compared with 25 and 15°C. All assays at different temperatures were performed in dried moving air, so even though the WLR was significantly greater at higher temperatures, the correction for the water vapour saturation deficit resulted in a lower CP at 35°C. This unexpected result could not be explained as a consequence of dehydration because assays were short-term recordings (about 30–35 min). It could also not be explained by better control of the spiracles (Schimpf et al., 2009; Wigglesworth, 1972) because we distinguished between RWL and CWL, and the lower CP at 35°C is observed both on GCP and corrected CP, i.e. calculated only from CWL. However, because of the short duration of the recordings, a possible explanation for the lower CP at the higher temperature could be the high initial rate of water loss attributable in part to the moisture adsorbed by the highly hygroscopic surface of the cuticle that lasts the entire recording at lower temperatures (15 and 25°C in our case). A faster rate of water loss during the first part of the recordings than during the last part was observed in many insects including: beetles (Schilman et al., 2008), locusts (Loveridge, 1968), *Drosophila* (Lighton and Schilman, 2007; Schilman et al., 2011), cockroaches (Gray and Chown, 2008) and ants (Lighton et al., 2004; Schilman et al., 2005). A similar abnormal relationship between saturation deficit and rate of water loss was found in locusts at 30°C, where the curvilinear relationship fell away from expected values at high saturation deficits (Loveridge, 1968). This resulted in a saving of between 1.5 and 2.5 mg of water per locust per hour at 25% relative humidity (RH) and between 2.7 and 4.0 mg at 0% RH. The anomalous relationship between saturation deficit and rate of water loss could be explained by shrinkage of the cuticle because of a quick initial water loss from the cuticle and the concomitant decrease in intermicellar pore dimension, reducing the water diffusion rate. No matter which is the mechanism underlying our observations, it does result in a substantial reduction in transpiratory water loss through the cuticle at high saturation deficits, and may be of considerable significance for conserving water reserves at times when a reduction in water loss is important.

RWL

The contribution of RWL to the significant drop of total water loss was apparent only after the 5th day post-feeding. This phenomenon is related to the significant decrease of CO_2 emission rate, simultaneously measured with WLR, from day 13 after feeding. RWL rates varied with temperature and starvation consistent with $\dot{V}_{CO_2}$. Considering desiccation tolerance alone, a drop of 0.1 mg h^{-1}

in RWL (e.g. the difference between 0 and 13 days post-feeding at 35°C; Table 2) in an insect of 50 mg (Table 1) with 35% of body mass as critical water content (Schilman et al., 2007) represents about two more weeks of survival. The significant decrease of metabolic and RWL rates with increasing starvation would work as an evolutionary strategy to survive in a changing environment with the uncertainty of finding a suitable host by saving both nutrients and water.

When we expressed the relative magnitudes of the different routes of water loss as a percentage of total water loss, RWL values were between 10% and 35%, depending on temperature and feeding state. These values are relatively high compared with values from the literature, mainly as a consequence of a highly waterproofed cuticle, as first stated by Zachariassen (Zachariassen, 1991) for a desert tenebrionid beetle and later discussed by Chown (Chown, 2002). Regarding the respiratory patterns, if we relate them to the water loss through the spiracles, we observe that a lower contribution from the respiratory route occurs on insects expressing cyclic gas exchange (mainly those insects measured at 15 and 25°C). A higher contribution of the respiratory water loss pathway occurs in insects measured at 35°C that express continuous gas exchange. There is a positive relationship between the RWL rate and the metabolic rate in *R. prolixus* males (note that this is required in order to apply the regression method). A similar positive relationship was previously found in five ant species (Schilman et al., 2005) as well as in species from two families of beetles; this correlation was stronger in species from dry than mesic environments (Zachariassen et al., 1987). Moreover, Woods and Smith (Woods and Smith, 2010) proposed a universal model which predicts that WLR scales to gas exchange with an exponent of 1 based on the results of 202 different species including 30 species of insects. The increase in RWL with increasing metabolic rate supports the hypothesis that species adapted to xeric environments have a lower standard metabolic rate compared with species adapted to mesic ones [e.g. the harvester ant *P. rugosus* (Lighton and Bartholomew, 1988)]. It also indirectly supports the idea of RWL reduction in species with DGE, although not necessarily as a consequence of the pattern itself, but as previously observed in *R. prolixus*, the change in respiratory pattern occurs by variation in metabolic rate (Contreras and Bradley, 2010). Higher temperatures increase metabolic rate and spiracles remain open during longer periods, resulting in a continuous pattern. On a mechanistic hypothesis, the DGE pattern could be explained by a reduction in brain activity for energy saving and delegating the control of spiracle opening to thoracic and abdominal ganglia (Matthews and White, 2011).

We think that RWL in insects has been underestimated as being a small component of total water loss, but it is very important for a small insect trying to survive in arid environments. Thus, more comparative studies focusing on the importance of RWL (e.g. Chown and Davis, 2003) should be encouraged in order to appreciate the real importance and processes of selection to reduce the spiracular component of WLR in insects, especially in small ectotherms, such as insects, whose metabolic rate and WLR are more susceptible to increasing temperature and declining rainfall, as predicted in many regions because of global warming (Chown, 2011; Chown et al., 2011).

MATERIALS AND METHODS
Animals
Adult males of *R. prolixus*, 20–30 days post-ecdysis, were used throughout the study. The insects were reared in the laboratory at 28°C on a 12 h:12 h light:dark cycle (lights on at 08:00 h) and they were fed weekly on live hens.

Respirometric measurements were performed at fixed intervals during a total period of 29 days and between measurements, the insects were kept at rearing temperature and light:dark conditions.

Respirometry
We used flow-through respirometry to measure real-time water vapour emission and CO_2 production in unrestrained adult males of the haematophagous bug *R. prolixus*. For all measurements, we used the high-resolution TR-2 Sable System International (SSI, Las Vegas, NV, USA) flow-through respirometry system (Lighton et al., 2004; Schilman et al., 2005). Briefly, air free of CO_2 and H_2O was drawn at a flow rate of ~55 ml min^{-1} by a SS4 sub-sampler (SSI), which unites a pump, needle valve and linearized mass flow meter, through low-permeability, Bev-A-Line tubing (to minimize errors associated with CO_2 and water vapour absorbance) and a RC-M precision miniature respirometer chamber (volume ~13 ml; SSI). The time response was less than 15 s. The water vapour and CO_2 produced by the haematophagous bugs were measured by a SSI RH-300 water vapour analyser (set to measure water vapour density in a range of 0 to 10 µg ml^{-1} and 0.0001 µg ml^{-1} of resolution) and a Li-Cor (LI-6251) CO_2 infrared analyser (Lincoln, NE, USA; resolution 0.1 ppm CO_2), respectively. Specimen temperatures were controlled to 15, 25 or 35°C by a SSI Pelt-5 temperature controller and SSI PTC-1 Peltier Effect cabinet. In order to equilibrate the temperature of the respirometer chamber with that inside cabinet, the air flow passed through a copper coiled tube (~6.5 m long) placed inside the cabinet. In addition, the activity of the insects was simultaneously monitored and recorded by an AD-2 activity detector (SSI) and the temperature measured by a thermocouple attached to a SSI TC-2000 thermocouple meter (accuracy 0.2 and resolution 0.01°C). The analog outputs from the analysers measuring CO_2, water vapour, insect activity, temperature of the chamber and air flow rate were connected to an A/D converter (SSI UI-2, 16 bit basic accuracy 0.05%) and stored on a computer by ExpeData data acquisition software (SSI).

Previous to the measurements, both CO_2 and water vapour analysers were calibrated. The CO_2 analyser was zeroed with nitrogen and spanned at 97±5 ppm with a certified span gas (Grupo Linde Gas SA, Buenos Aires, Argentina). The water vapour analyser was zeroed with nitrogen and spanned by bubbling air through pure water at an accurately known temperature (measured by a thermocouple attached to a TC-2000) ~5°C lower than room temperature. The RH-300 was set to its dew point mode and adjusted to read the correct water temperature, i.e. temperatures reading from the TC-2000 and RH-300 matched.

Measuring RWL: water loss regression method
We analysed the data with the regression technique developed by Gibbs and Johnson (Gibbs and Johnson, 2004). This method is useful because it allows the estimation of RWL in insects performing continuous gas exchange. Briefly, we plotted WLR against CO_2 release for each individual insect using all values over 10 min of the last part of respiratory recording. Extrapolation to the intercept provides an estimate of corrected cuticular water loss, i.e. without the spiracular component. The slope of each regression line estimates the hygric cost of gas exchange for that recording, i.e. the incremental increase in water loss associated with CO_2 release. RWL is calculated with the equation:

$$RWL = RS[CO_2], \qquad (1)$$

where RWL is estimated by the regression method (Gibbs and Johnson, 2004), RS is the slope of the regression expressed in mg H_2O h^{-1}/µl CO_2 h^{-1}, and CO_2 is the $\dot{V}_{CO_2}$ in µl CO_2 h^{-1}. For a detailed explanation of the method, see Gibbs and Johnson (Gibbs and Johnson, 2004).

Experimental procedure
We identified the insects by painting a colour code on their legs with acrylic paint, and weighed them individually to the nearest 0.1 mg using an analytical balance (Mettler AJ100, OH, USA). Insects were placed in a communal jar for feeding and 4 h later they were weighed again. It is known that during the first 3 h after feeding, *R. prolixus* eliminate most of the excess water from the blood meal (Maddrell, 1964). Therefore, in our results we excluded differences in mass loss due to different rates of removal of
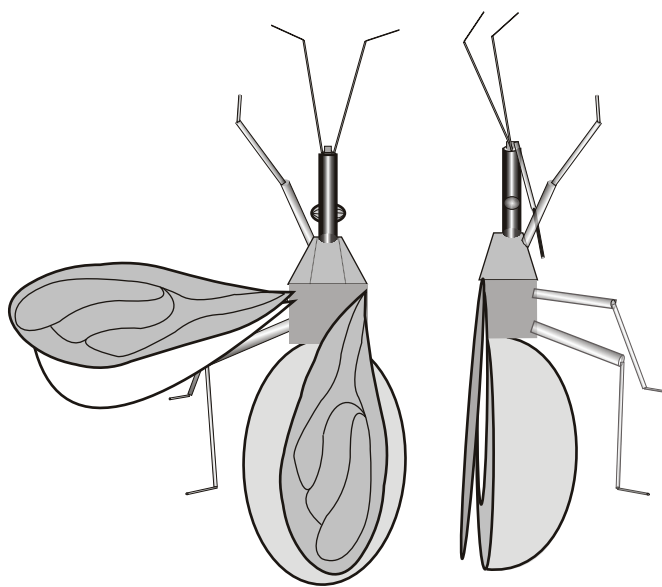
Fig. 5. Diagram of the geometric figures used to estimate body surface area. *Rhodnius prolixus* is shown in the dorsal (left) and lateral (right) view.

$$TSA = A_{head} + A_{rostrum} + 2[A_{antenna}] + 2[A_{legI}] + 2[A_{legII}] \\ + 2[A_{legIII}] + 4[A_{tegmen}] + 4[A_{wing}] + A_{dorsal\,thorax} \\ + 2[A_{lateral\,thorax}] + A_{ventral\,thorax} + A_{dorsal\,abdomen} \\ + A_{ventral\,abdomen}, \quad (2)$$

$$WSA = A_{head} + A_{rostrum} + 2[A_{antenna}] + 2[A_{legI}] + 2[A_{legII}] \\ + 2[A_{legIII}] + A_{dorsal\,thorax} + 2[A_{lateral\,thorax}] + A_{ventral\,thorax} \\ + A_{dorsal\,abdomen} + A_{ventral\,abdomen}. \quad (3)$$

Surface area decreased with starvation mainly through changes in abdomen surface area, with a fixed factor related with insect size (supplementary material Fig. S3).

We applied a mixed-effects regression model for longitudinal data (supplementary material Fig. S4). Two models were performed to estimate insect surface area: model I for wingless body surface area (Eqn 4) and model II for total body surface area (Eqn 5) (including both side surface area wings) (see below).

The intercept and slope population parameters represent the overall (population) trend, while the individual parameters express how subjects deviate from the population trend (Hedeker and Gibbons, 2006).We used the population parameters to predict the surface area of insects, because this is the average across the individuals. To control for inter-individual variability in the model, we included the length of tibia 3 (Ti3) for each individual. Insect squared mass was found to be a good predictor of time after feeding (correlation index: −0.84).

Selection of the model
Model selection was made using Akaike's information criterion and the Bayesian information criterion. With these criteria, the model with a lower value has a better fit (Singer and Willett, 2003). Nested model comparisons were performed by maximum likelihood (Singer and Willett, 2003). In both cases, the incorporation of squared mass, the Ti3 variables and the random effect markedly reduced fitting indicators. The final model was fitted by restricted maximum likelihood.

Estimated parameters were significantly different from zero ($P<0.001$); nonetheless, the parameter associated with the Ti3 only for model I is marginally significant ($P=0.068$). We used the nlme package (Pinheiro et al., 2013; R Development Core Team, 2013). As a result of this analysis we obtained the formulae used to estimate the body surface for model I:

$$WSA = 146.36 + 3.01 x 10^{-3} Mass^2 + 10.50 Ti3, \quad (4)$$

and model II:

$$TSA = 237.88 + 2.94 x 10^{-3} Mass^2 + 32.08 Ti3, \quad (5)$$

where body mass is expressed in mg, the length of Ti3 is expressed in mm, and WSA and TSA are expressed in mm^2.

Analyses and statistics
Respirometry data were stored on a laptop computer and analysed by ExpeData data acquisition and analysis software (SSI). The following corrections and conversions were made from the recordings. (1) CO_2 and H_2O baselines were subtracted assuming a linear drift. (2) CO_2 in ppm was converted to $\mu l\,h^{-1}$ [for formulae, see Lighton (Lighton, 2008)]. (3) H_2O vapour density in $\mu g\,ml^{-1}$ was converted to WLR in $mg\,h^{-1}$ (by multiplying by flow rate in $ml\,h^{-1}$). (4) The CO_2 and water vapour signals were lag corrected because they were slightly out of phase as a result of the experimental arrangement, i.e. analysers were arranged in series, thus the air coming out of the respirometry chamber arrived first at the H_2O and then at the CO_2 analyser. (5) The activity signal (in volts) was copied again, into another empty channel, and its ADS was calculated. The ADS is the cumulative sum of the absolute difference between all of the adjacent data points. The ADS was originally used as a means of translating bi-directional position measurements into an accumulated displacement vector (Lighton et al., 1993a), but has proved to be of broader utility as a measure of the short-term dynamic variability of data (e.g. Lighton and Turner, 2004).

After corrections and conversions were made, the following values were measured and analysed from the recording: (1) mean values of CO_2 and H_2O from the last 20 min of the recording and (2) the range (difference between

redundant water immediately after feeding. We discarded insects that did not feed because we wanted to analyse the effect of starvation on metabolic rate and WLR. Each insect was randomly assigned to a temperature treatment (15, 25 and 35°C) and respirometric and mass measurements were performed at different times after feeding (0, 5, 13 and 29 days). Simultaneously, 12 fed insects of the same batch were used for surface area estimation. Surface area was calculated for each post-feeding interval tested in respirometric assays (see 'CP and surface area estimation', below, for a description of the method).

Each assay began with 3–5 min of baseline recording, which was paused before the insect was placed inside the chamber. After 10 min, to allow the system to stabilize, recording was resumed and lasted ~25 min; then, the recording was paused again, the insect was removed from the chamber and the final baseline was recorded.

CP and surface area estimation
To obtain CP, we measured WLR and estimated the surface area and its variation as a function of feeding state at 0, 5, 13 and 29 days after feeding. Twelve specimens were photographed (Nikon S6300) in the dorsal, lateral and ventral view, weighed to the nearest 0.1 mg and the abdomen maximum thickness measured with a digital calliper. The data set includes only those individuals for which all variables could be measured at all four times.

Fig. 5 shows a scheme of the geometric shapes we used to calculate the surface area of insects. The head was approximated to the surface area of a cylinder minus the surface area of the ellipses and adding the corresponding ellipsoid surface areas of the eyes. The surface area of the antennae and rostrum was calculated as the sum of two cylinders. Each leg was constructed as the sum of three cylinders while the thorax was taken as the sum of a trapezium (anterior region) and rectangle (posterior region).The abdomen surface area was constructed as the area of an ellipse (dorsal) and ellipsoid (lateral). Finally, the left wing was digitally photographed and its surface area calculated with morphometric software (TPSdig, version 1.39). We also used this software to obtain other magnitudes, such as the length and width of the thorax and abdomen in the dorsal and ventral views, respectively. After the 29th day post-feeding, specimens were killed and the rest of the measurements were performed using a Leica MZ8 stereomicroscope (Wetzlar, Hesse, Germany) with a graduated ocular. The total (TSA) and wingless (WSA) surface area were calculated as the sum of the individual surface areas described above (for median and confidence intervals of calculated surfaces, see supplementary material Table S2):

maximum and minimum values of the activity ADS from same last 20 min of recording). We saved these values in a spreadsheet for further data manipulations. The spreadsheet also included the water vapour saturation deficit from chamber temperature (see Lighton and Feener, 1989), the insect surface area and, hence, gross CP (i.e. combined RWL and CWL), the CP and the respiratory component of WLR calculated by the regression method (Gibbs and Johnson, 2004).

Means are accompanied by s.e. and sample sizes. The effect of temperature and feeding state on the measured variables was tested using repeated measures ANOVA. When required, the variables were transformed to meet the model's assumptions. Furthermore, when deviations from sphericity existed, the degrees of freedom were adjusted with the lower bound epsilon or we used a generalized least squares approach. Regressions are by least squares, with axis transformations where noted, and were tested for statistical significance by ANOVA. Regressions are compared by analysis of covariance (ANCOVA).

There are different approaches to establish an objective criterion to classify the continuous or discontinuous pattern of gas exchange (e.g. Marais et al., 2005; Shelton and Appel, 2000; Lighton et al., 1993b). Here, we categorized $\dot{V}_{CO_2}$ patterns using the method described by Marais et al. (Marais et al., 2005). Briefly, the percentage of points above the middle line of each trace was computed. Traces with less than 30% of the points above the middle line were considered cyclic. To analyse the effect of temperature and movement on respiratory pattern, we constructed a logistic generalized mixed model defining temperature as a factor and activity as a continuous explicatory variable (null model: AIC 195.97, with the chosen model AIC: 110.54).

At the same time, we calculated the VC to quantify the degree of discontinuity of CO_2 liberation on each recording (Lighton et al., 1993b; Shelton and Appel, 2000):

$$VC = \frac{s.d.}{Mean}. \tag{6}$$

A smaller VC portrays a continuous and more homogeneous pattern, where spiracles remain open and gas exchange is relatively constant. We tested the occurrence of cyclic gas exchange using temperature and nutritional state together with ADS as a proxy for activity, fitting a generalized linear mixed model with binomial distribution.

All data was analysed using Infostat Statistical software (Di Rienzo et al., 2011) and R version 3.0.1 (R Development Core Team, 2013).

Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.109298/-/DC1

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