Physiological Entomology

*Physiological Entomology* (2017), DOI: 10.1111/phen.12199

# Critical threshold meal size and molt initiation in *Rhodnius prolixus*

ROBERTO A. SAENZ[1], PAULA MEDONE[2], NATALIA DI CLEMENTE[3], ANTHONY TONGEN[4] and JORGE RABINOVICH[2]

[1]Facultad de Ciencias, Universidad de Colima, Colima, Mexico, [2]Centro de Estudios Parasitológicos y de Vectores (CEPAVE-CCT-La Plata-CONICET-UNLP), La Plata, Argentina, [3]Laboratorio de Biorremediación de Suelos, Centro de Investigación y Desarrollo en Fermentaciones Industriales, Universidad Nacional de La Plata, La Plata, Argentina and [4]Department of Mathematics and Statistics, James Madison University, Harrisonburg, Virginia, U.S.A.

**Abstract.** The molting process and body growth in *Rhodnius prolixus* (Hemiptera: Reduviidae) (Ståhl, 1859) are significantly influenced by the availability and quality of food. Based on the body weight of each stage, the present study provides estimates of a potential critical weight threshold required for molt initiation in *R. prolixus*. In addition, a new measure given by the area under the weight curve is proposed, which encapsulates both body weight and time. It is shown that this measure is consistent with the data, and allows the estimation of a pre-refractory period (i.e. the time interval between the moment at which the critical weight threshold is reached and the moment when no further meals are accepted). The present analysis estimates the critical weight threshold as 1.6, 5.3, 12.9, 42.0 and 97.0 mg for stages 1–5, respectively, whereas the values of the area under the curve threshold as 5, 16, 31.2, 159.7 and 329.9 mg days for stages 1–5, respectively. The results of the present study confirm the existence of a weight-dependent mechanism for the initiation of molting in *R. prolixus*.

**Key words.** Area under the curve, critical weight, feeding interval, feeding strategy, feeding time, haematophagy, meal size, molting initiation, refractory period, *Rhodnius prolixus*.

## Introduction

Similar to most arthropods, pre-adult nematodes and other Ecdysozoa, insects show a punctuated growth indicated by periodic molts, during which successive larger cuticles are formed to accommodate ongoing body growth. The body growth and molting processes are influenced by a number of environmental factors, most importantly the quantity and quality of food. In vector insect species, body weight and other feeding parameters (e.g. minimum food ingestion for molting, duration of feeding and initiation of molt after feeding) are important for population growth and persistence under variable and frequently uncertain host availability conditions, and thus they are also indirectly related to disease transmission.

Correspondence: Roberto A. Saenz, Facultad de Ciencias, Universidad de Colima, Bernal Díaz del Castillo 340, Col. Villas San Sebastián, C.P. 28045 Colima, Col., Mexico. Tel.: +52 312 3161135; e-mail: rsaenz@ucol.mx

*Rhodnius prolixus* (Hemiptera: Reduviidae) (Ståhl, 1859) is an important vector of Chagas disease that is distributed in Central America and northern South America, especially Venezuela, Colombia, Guatemala, Honduras and El Salvador. *Rhodnius prolixus* inhabits mainly wild environments, such as palm trees, although it is also well adapted to domestic environments (Schofield, 1994). *Rhodnius prolixus* is one of the best known triatomine species from a physiological point of view, from the initiation of food searching (orientation to the host) to feeding features (Schilman & Lazzari, 2004; Ferreira *et al.*, 2007; Fresquet & Lazzari, 2011; Vinauger *et al.*, 2012). From the earliest studies on blood ingestion in *R. prolixus* onward, the feeding process is described as a 'chain of reflexes' (Wigglesworth & Gillett, 1934) in which the initiation of the response is conditioned by the physiological state of the insect (Friend & Smith, 1977) and the insect starts seeking food via the perception of the radiant heat of a warm source at a distance (Lazzari & Núñez, 1989), with the antennae

© 2017 The Royal Entomological Society

playing a key function (Wigglesworth & Gillett, 1934; Flores & Lazzari, 1996). In *R. prolixus*, all five nymphal instars and adults are obligatory hematophagous insects and potential vectors of *Trypanosoma cruzi*, the aetiological agent of Chagas disease, which afflicts 6–7 million people worldwide, mostly in Latin America (WHO, 2016). Similar to most triatomines, *R. prolixus* is unable to molt without feeding and nymphs usually have to feed more than once to be able to molt to the next stage. Individuals that have passed a 'critical period' after feeding produce a factor or hormone that induces molting (Wigglesworth, 1934). However, there is no information available on the relationship between minimum blood meal size ingested and time from blood ingestion to molt initiation. Various relationships have been investigated between weight, weight gain and molting in *R. prolixus* (J. E. Rabinovich, P. Medone & N. di Clemente, unpublished data), although no attempt has been made to tackle the 'critical period', nor the minimum blood meal size ingested that triggers molt initiation.

In other insect species, the existence of an inverse relationship between body growth rate and the timing of the next molt is taken as evidence that molt does not occur on a regular time schedule but instead is triggered by a growth- or size-dependent mechanism; this relationship is theoretically analyzed by Hentschel (1999). In the tobacco hornworm larvae *Manduca sexta*, molt is triggered when larvae reach a well-defined 'critical weight' (Nijhout & Williams, 1974a, 1974b; Davidowitz *et al.*, 2003, 2004; Nijhout *et al.*, 2006; Callier & Nijhout, 2011). The critical weight is defined as the weight (or body size) at which an insect larva makes the developmental-physiological 'decision' to begin the molting process. At the critical weight, a developmental process is initiated that culminates in the secretion of ecdysone, which causes the cessation of feeding and the molt to occur.

By contrast to Lepidoptera such as *M. sexta*, triatomines have a limited feeding time on their vertebrate hosts because of predation risk by their host. Accordingly, it is not clear whether a threshold weight is responsible for initiating the molting process in triatomines. The present study aims to determine the critical threshold body weight or meal size required to initiate molting for each nymphal stage of *R. prolixus*, feeding on a restrained live mouse, thus eliminating the predation risk. An estimate of an additional physiological parameter that we term the 'pre-refractory period' (i.e. the time between reaching a critical body weight threshold and the cessation of feeding) is also provided. In our analysis, four potential triggers for molting are considered: body weight, amount of ingested blood, relative gain of weight, and a combination of weight and time. The implications of the results of the present study are discussed in light of evolutionary theory.

## Materials and methods

### *Laboratory methodology*

An experiment was designed where the feeding frequency and the feeding time of different *R. prolixus* stages were controlled. Single individuals of each of the five instars of *R. prolixus* were fed on a mouse (using a different individual mouse on each occasion); 10 replicates were used for each stage, except for instar 1, for which only five replicates were used. Individuals were fed for fixed times ranging from 2 to 12 min, combined with a given time interval among meals determined by the experimenter (every 2, 4, 6, 8 or 10 days); the selected intervals cover previous estimates of the average number of days between meals under field domiciliary conditions (Rabinovich *et al.*, 1979). Each individual was offered a blood meal for 2 and 4 min in stages 1, 2 and 3; for 4 and 8 min in stage 4; and for 4, 8 and 12 min in stage 5. For all stages, insects were kept and exposed individually to all combinations of feeding durations and intervals between meals. All insects were weighed individually before and after the host was offered for feeding; the weight after feeding was taken immediately after the interruption of the meal to ensure that no diuresis took place; all individuals were checked daily to record the exact date of death or molting. After an individual had been used in a given experiment, it was returned to the general rearing colony and never used in the same or any other experiment. Individuals who died before molting and those who molted without rejecting any meal were removed from the analysis (the latter could not be analyzed with the employed methodology; an explanation for this is below). The number of individuals analyzed was 33, 67, 89, 98 and 124 for stages 1, 2, 3, 4 and 5, respectively. The variation in the number of individuals analyzed from each stage was a result of differences between stages in the combination of feeding frequency and feeding time, as well as other factors (e.g. removal from the analysis of those individuals that molted without rejecting a meal, and discarding individuals that died or had an abnormal molt). All individuals used in the experiments were taken from the general *R. prolixus* colony, without any particular selection criteria; because the jars of the colony containing the insects were periodically mixed, we assume that the individuals used in the experiments can be considered as a real Mendelian population and are only randomly related. Controlled rearing conditions were kept constant (26 °C and 60% relative humidity); more details on the laboratory methodology are available elsewhere (J. E. Rabinovich, N. di Clemente & P. Medone, in preparation).

### *Triggers of molting*

Two physiological indicators were used to estimate a threshold value that triggers molting. The first was exclusively dependent on body weight and the second was dependent on both body weight and the time an individual has spent in a given stage after the first offered meal. Because there is no clear significant pattern for feeding frequency and feeding time on molting probability and time (J. E. Rabinovich, P. Medone & N. di Clemente, unpublished data), it was assumed that these factors do not impact the initiation of molting. More importantly, it was assumed that, for a given indicator, there is a threshold value such that, if the level of the indicator reached this threshold, then the molting process would initiate. In addition, the goal was to identify a measure that did not depend on any specific feeding patterns because these would be unknown outside

an experimental setting. Therefore, the data for each stage, corresponding to different feeding lengths and frequency, were pooled together for their analysis. For example, for stage 1, all 33 individuals were pooled to determine the physiological indicator associated with the trigger of molting.

*Weight-related measures.* Three measures were defined as potential triggers of molting that depend only on body weight: (i) total body weight (mg); (ii) meal size as amount of blood ingested (mg, as the difference between pre- and post-feeding recorded weights); and (iii) relative body weight (total body weight relative to the initial body weight before the first meal, i.e. a dimensionless number). Each of these measures was given as a function of time; because weight was recorded in fixed time intervals, a linear interpolation between recorded body weights was used (lines joining the points in Fig. 1A) to obtain a continuous curve for the change in time of the above measures.

*Body weight and time: area under the curve (AUC).* The AUC of the weight was employed as a measure that depends both on body weight and time subsequent to the beginning of the experiment; by definition, the units of AUC are mg days, representing the accumulated body weight in a given time interval (from the beginning of the experiment until a given time). The complete weight curve was created by means of a linear interpolation between recorded weights at several time points determined by the time intervals of the experimental design. The area was computed numerically for each individual using the trapezoidal method for estimating the integral of a function. The resulting AUC was a function of time from zero (at the initial weight of the insect before the first meal was offered) until the time the first meal is rejected because, by our definition of threshold (see below), a rejected meal was a sign that the threshold had been achieved. This explains why all individuals that molted without rejecting any offered meal were excluded from our analysis (otherwise, there would not be an end point for interpolating the last interval). On this basis, a total of 7, 33, 7, 0 and 2 insects from stages 1, 2, 3, 4 and 5, respectively, were removed from the analysis. A graphical explanation of these definitions is provided in Fig. 1(B, C).

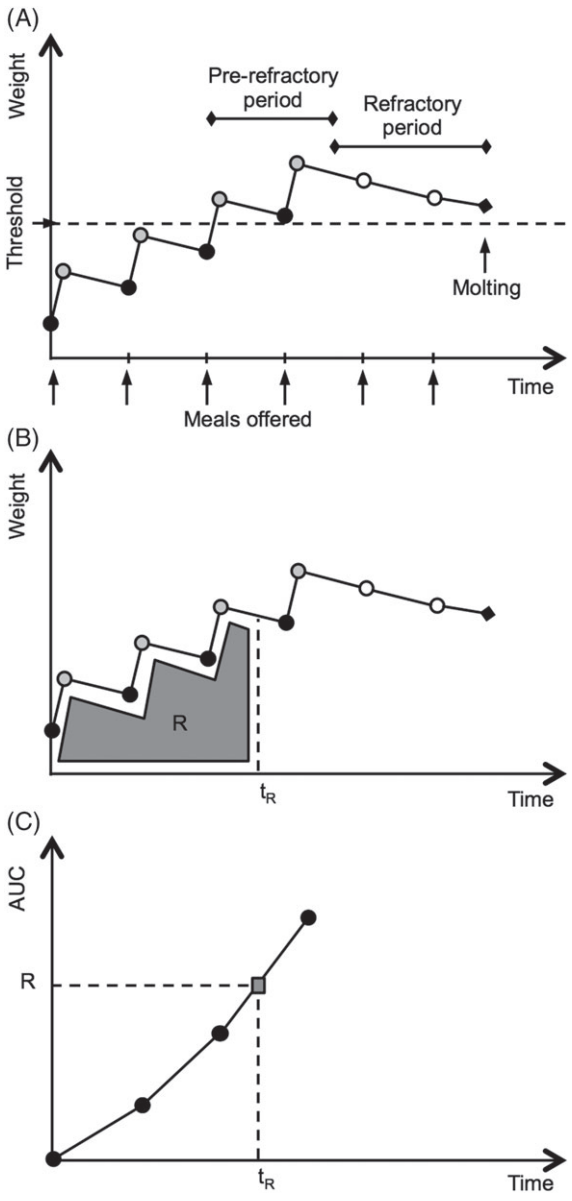


**Fig. 1.** The key concepts in the molting process. (A) The concepts of threshold, pre-refractory and refractory periods based on a weight curve as the trigger for the molting process. Black and white circles represent the insect weight when an offered meal is accepted or not accepted, respectively. Grey circles represent the insect weight after feeding. (B) Weight as a function of time. Shaded region represents the area $R$ under the curve from the initial time (start of experiment) until time $t_R$. (C) Area under the weight curve (AUC) as a function of time. Area of region $R$ in (B) is just a single point $(t_R, R)$ in the AUC curve.

*Threshold values*

The threshold was defined as the value of any of the above indicators that, once reached, initiates the physiological processes leading to triatomine molting.

To estimate the threshold value for any specific measure, the number of individuals that reached each feasible value of the threshold (i.e. those values that are candidates to be the threshold, which vary for each specific measure and each insect stage) was recorded. Such individuals were referred to as 'valid individuals'. The 'molting period' was defined as the period of time elapsed from the moment the threshold was reached until molting was complete. It was assumed that the best estimate of the threshold was the greatest value that corresponded to the largest number of valid individuals and the minimum variance of the molting period. Ideally, all individuals should be valid, which would correspond to the simplest estimation of the threshold value. A weight was required to rescale the two quantities that were being optimized (maximum number of valid individuals and minimum variance of the molting period) because they had different dimensions. An arbitrary weight of 10% of the variance of molting period was chosen, which implied that a largest

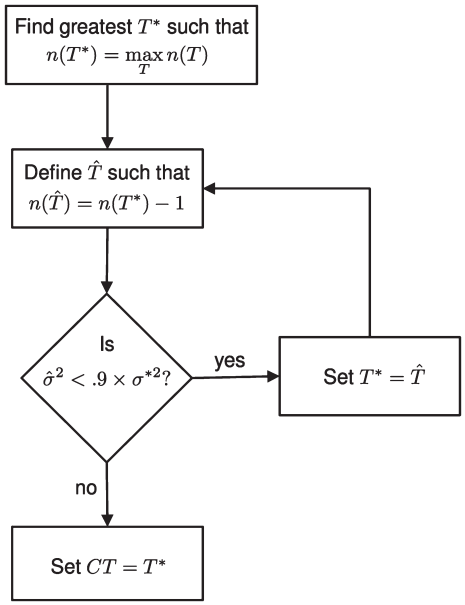


**Fig. 2.** Flowchart describing the algorithm for the computation of the best estimate of the threshold,*CT*. *T* denotes a value of a given measure that defines the threshold, $n(T)$ is the number of valid individuals that satisfies the threshold definition for the measure value *T*, and $\sigma^2$ denotes the variance of the molting period associated to a specific value of the measure *T*.

threshold value was selected with one less valid individual only if there was a decrease of at least 10% in the variance of the corresponding molting period. A flowchart of the algorithm used to compute the best estimate for the threshold is provided in Fig. 2: the greatest value of any of the four measures of the threshold *T* that maximized the number of valid individuals, called $n(T)$, was searched for; this value *T* was called *T**; then the value of the measure $\hat{T}$ that was satisfied by one less individual than *T** was selected if its corresponding variance had a reduction of at least 10% (from $\sigma^{*2}$ to $\hat{\sigma}^2$) (i.e. if $\sigma^{*2} < 0.9 \times \hat{\sigma}^2$); in that case, *T** was changed to $\hat{T}$ and the previous step was repeated, otherwise the best estimate threshold *CT* was set at $CT = T^*$.

*Pre-refractory, refractory and molting periods*

The 'refractory period' was defined as the interval between the time when no more blood was ingested (i.e. no more meals would be accepted) from a series of successive food offers, until ecdysis took place. Conversely, the 'pre-refractory period' was defined as the time interval from the moment the threshold was reached until the time when no more blood was ingested (no more offered meals would be accepted). The pre-refractory period is similar to the 'interval to cessation of growth' or 'terminal growth phase' used by Callier & Nijhout (2013) when referring to a similar process in *M. sexta*. The 'molting period' was defined as the sum of pre-refractory and refractory periods. A graphical description of these definitions is provided in Fig. 1(A).

Because the date of ecdysis was recorded in the experiments, estimating the time when the threshold is reached provided a direct estimation of the molting period; thus, an estimate for the pre-refractory period determined an estimate for the refractory period (and vice versa). The pre-refractory period was estimated using the best estimate for the threshold value. A feasible value for the pre-refractory period was tested by counting the number of individuals for which the data were consistent with that threshold value; an individual was consistent with the threshold value if the pre-refractory period being tested was between the computed bounds from the experimental data, where the lower bound was the period between time at threshold and the time for the last accepted meal, and the upper bound was the period between the time at threshold and the time when a meal was rejected for the first time, and thereafter always rejected until molting. Among the possible values of the pre-refractory period, the values with the largest number of valid individuals were preferred. To cover the whole range of feasible values for the pre-refractory period, 1-h intervals were taken for each (computational) test (covering a range between the interval [0,1], which corresponded to the first hour, up to the interval [167,168], which corresponded to the hour just before a reasonably upper arbitrary value of 7 days).

## Results

*Weight-related measures*

Table 1 shows the estimated threshold value for each nymphal stage using the absolute body weight as the measure that triggers molting. Table 1 also includes the number of valid individuals and the variance and mean of molting period corresponding to the best estimate of the threshold values. Threshold values are fulfilled for almost 100% of individuals in most stages. The mean molting period ranges from 7.8 days for stage 1 to 12.4 days for stage 4, increasing steadily for the first four stages by an average of 1.5 days per successive stage. The mean molting period for stage 5 is 21.5 days (i.e. there is an abrupt increase of 9 days from the previous stage). However, this abrupt increase in the mean molting period for stage 5 was not reflected in its estimated threshold value, which follows the smooth pattern of increase from the previous stages (an increase factor between 2 and 3 per stage). The results obtained when using the change in body weight or the relative body weight

**Table 1.** Best estimates for threshold values by stage, with the corresponding number of valid individuals (see text) and molting period mean and variance, based upon the absolute (total) body weight as the measure that triggers the molting process.

| Stage | Best estimate of insect total weight threshold value (mg) | Number of valid individuals (%) | Mean molting period (days) | Variance of molting period ($days^2$) |
|---|---|---|---|---|
| 1 | 1.6 | 28 (84.9%) | 7.89 | 1.36 |
| 2 | 5.3 | 61 (91.0%) | 9.49 | 1.25 |
| 3 | 12.9 | 87 (97.8%) | 10.52 | 2.88 |
| 4 | 42.0 | 98 (100.0%) | 12.47 | 1.43 |
| 5 | 97.0 | 123 (99.2%) | 21.53 | 21.63 |

**Table 2.** Best estimates for insect total blood ingested threshold values by stage, with the corresponding number of valid individuals (see text) and molting period mean and variance, based upon the change in body weight (blood ingested) as the measure that triggers the molting process.

| Stage | Best estimate of insect total blood ingested threshold value (mg) | Number of valid individuals (%) | Mean molting period (days) | Variance of molting period (days$^2$) |
|---|---|---|---|---|
| 1 | 1.2 | 31 (93.9%) | 8.10 | 1.49 |
| 2 | 3.4 | 61 (91.0%) | 9.44 | 1.35 |
| 3 | 7.0 | 84 (94.4%) | 10.35 | 1.89 |
| 4 | 23.0 | 98 (100%) | 12.47 | 1.43 |
| 5 | 49.0 | 123 (99.2%) | 21.67 | 23.08 |

**Table 3.** Best estimates for threshold values by stage, with the corresponding number of valid individuals and molting period mean and variance, based upon the relative weight (weight relative to the initial weight before the first meal) as the measure that triggers the molting process.

| Stage | Best estimate of relative weight threshold value (dimensionless) | Number of valid individuals (%) | Mean molting period (days) | Variance of molting period (days$^2$) |
|---|---|---|---|---|
| 1 | 2.3 | 30 (90.9%) | 8.43 | 2.86 |
| 2 | 1.7 | 62 (92.5%) | 9.45 | 1.53 |
| 3 | 1.9 | 86 (96.6%) | 10.48 | 2.06 |
| 4 | 2.4 | 97 (99.0%) | 12.40 | 1.70 |
| 5 | 1.2 | 124 (100%) | 23.96 | 23.86 |

as the measure for triggering the molting process are shown in Tables 2 and 3, respectively.

Figure 3 shows the variation of the percentage of valid individuals per instar, as well as the variance and mean of the molting period, when different values are selected for the threshold. In Fig. 3, the best estimate of the threshold value for each instar is shown as a blue vertical line (representing the threshold associated with the largest number of valid individuals together with minimum variance of the molting period). The number of valid individuals decreases as the threshold value is increased (black solid curves in Fig. 3). On the other hand, the behaviour of the variance of the molting period varies from stage to stage as the threshold value is increased; in some cases showing a clear local minimum, whereas, in others, it decreases with the threshold value (red dashed curves in Fig. 3). Similar patterns were found for curves corresponding to Table 2 (using blood ingested as the indicator measure) and Table 3 (using relative weight as the indicator measure) that are analogous to Fig. 3 (figures not shown).

*Body weight and time: AUC*

The best estimates for the threshold value of each stage, using the AUC as an indicator of the trigger for the molting

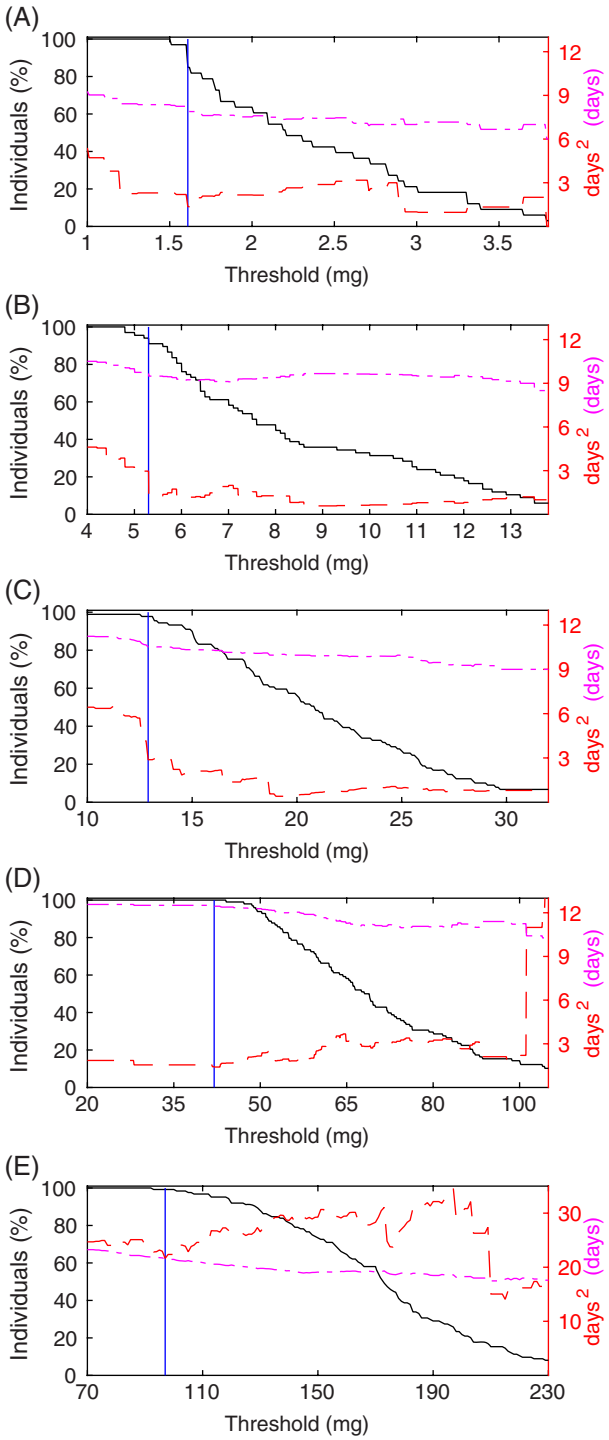


**Fig. 3.** Results based on the use of the absolute (total) body weight as the trigger for the molting process. Percentage of valid individuals (solid curve; black), variance of molting period (dashed curve; red) and mean of molting period (dot-dashed curve; magenta) for each threshold value for (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5. The horizontal axis shows the range of feasible values for the measure that was considered for each stage. The best estimate of the threshold value that triggers molting is shown as a solid blue vertical line. [Colour figure can be viewed at wileyonlinelibrary.com].

**Table 4.** Best estimates for threshold values by stage, with the corresponding number of valid individuals and molting period mean and variance, based upon the area under the data curve as the measure that triggers the molting process.

| Stage | Best estimate of threshold value (mg days) | Number of valid individuals (%) | Mean molting period (days) | Variance of the molting period (days$^2$) |
|---|---|---|---|---|
| 1 | 5 | 33 (100%) | 6.90 | 1.85 |
| 2 | 16 | 67 (100%) | 8.36 | 3.26 |
| 3 | 31.2 | 88 (98.9%) | 9.43 | 5.27 |
| 4 | 159.7 | 98 (100%) | 9.82 | 1.59 |
| 5 | 329.9 | 124 (100%) | 21.10 | 21.25 |

process, are shown in Table 4. Such values increase as the insects develop through their five stages, ranging from 5 mg days in stage 1 to 329.9 mg days in stage 5. The number of valid individuals used in the analysis that is consistent with such threshold value estimates, as well as the mean and the variance of its corresponding molting period, is shown in Table 4. In all stages, all of the individuals in the analysis are valid (except for stage 3, with only one individual not valid). The mean molting period also increases per successive stage, ranging from 6.9 days for stage 1 up to 21.1 days for stage 5. However, the results for stage 5 should be considered with caution because the variance for the molting period is as large as its mean, as was also found for weight measures (Tables 1–3).

Figure 4 shows the variation of the percentage of valid individuals, as well as the variance and mean of the molting period, for different assigned threshold values when the threshold value is allowed to vary (for all stages); the best estimates for threshold values are shown as a blue vertical line for each stage. The number of valid individuals decreases as the threshold value is increased (black solid curves in Fig. 4); however, the behaviour of the molting period variance varies between stages as the threshold value is increased; in some cases showing a clear local minimum, whereas, in others, it decreases with the threshold value (dashed red curves in Fig. 4).

Note that the AUC is defined in terms of the total body weight. However, the AUC approach could be also easily implemented with respect to the change in weight or other relative weight measures. The analysis was only focused on total body weight because similar values for best estimates of molting period were obtained for the other measures (Tables 1–3).

*The pre-refractory period*

Given the best estimate for the AUC threshold for each stage (Table 4), the number of individuals consistent with several pre-refractory periods was estimated (Fig. 5). For example, in stage 1, there are 31 individuals (93.9%) consistent with a 1-day pre-refractory period, although there are only three individuals (9.1%) consistent with a 5-day pre-refractory period (Fig. 5A). The intervals containing the values for the pre-refractory period, as associated with the top 5% largest number of valid individuals, are indicated in Fig. 5 by lines below and above the

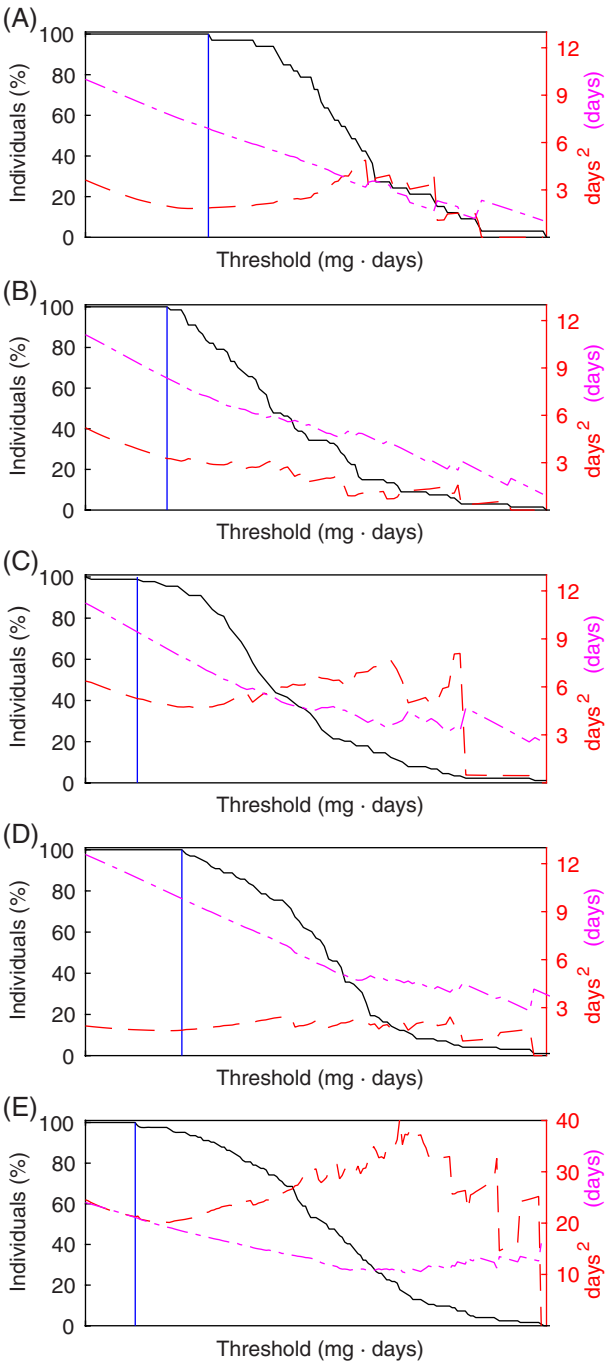


**Fig. 4.** Results based on the use of the area under the weight curve as the trigger for the molting process. Percentage of individuals (solid curve; black) and mean (dot-dashed curves; magenta) and variance (dashed curves; red) of the molting period for each threshold value for (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5. The horizontal axis shows the range of feasible values for the measure that was considered for each stage. The best threshold AUC estimate in each case is shown as a solid blue vertical line. [Colour figure can be viewed at wileyonlinelibrary.com].

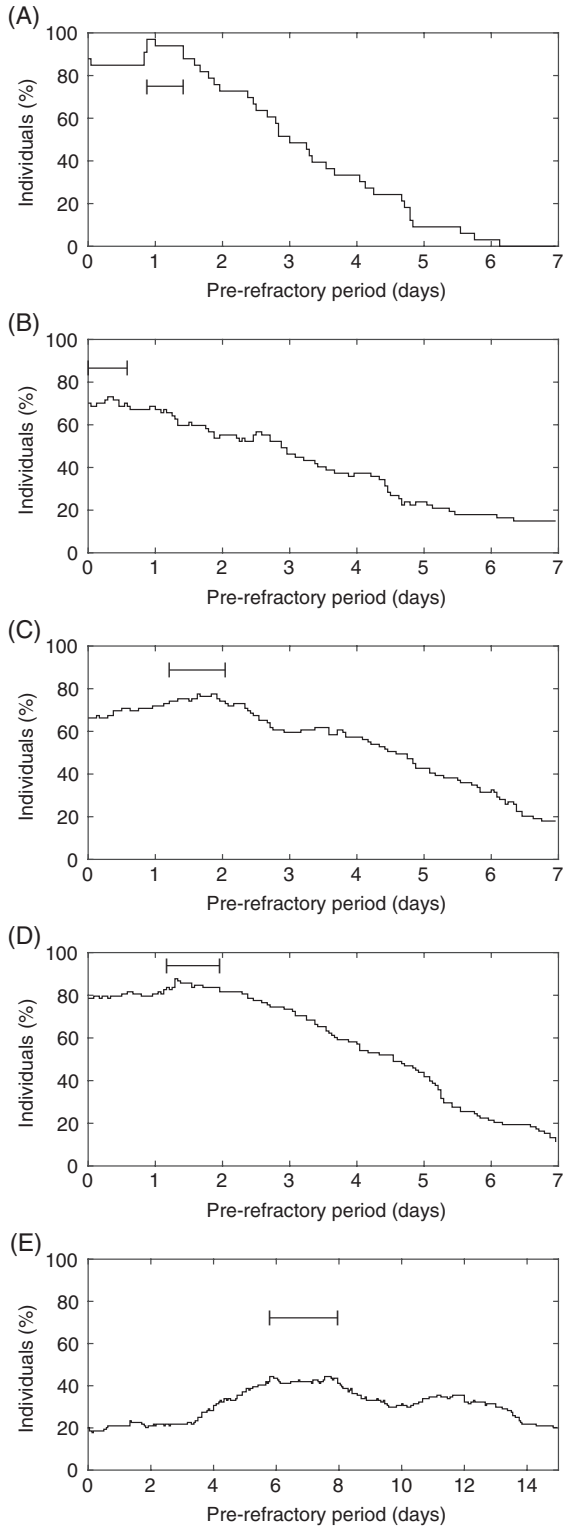


**Fig. 5.** Percentage of individuals consistent with the pre-refractory period for (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5. The line above the curve shows the interval of values for the pre-refractory period associated with the top 5% largest number of valid individuals.

**Table 5.** Estimated intervals for the pre-refractory, refractory and molting periods for each stage.

| Stage | Pre-refractory period (days)[a] | Refractory period (days)[b] | Molting period (days)[c] |
|---|---|---|---|
| 1 | 0.9–1.4 | 5.5–6.0 | 6.4–7.4 |
| 2 | 0–0.6 | 7.9–8.2 | 7.9–8.8 |
| 3 | 1.2–2.0 | 7.8–7.9 | 9.0–9.9 |
| 4 | 1.2–2.0 | 8.1–8.1[d] | 9.6–10.1 |
| 5 | 5.8–8.0 | 13.9–13.9[d] | 20.3–21.9 |

[a]Interval for the pre-refractory period that contains the top 5% largest number of valid individuals for each stage.
[b]Computed as the difference between the molting period and the pre-refractory period.
[c]95% confidence interval using mean, variance and sample size from Table 4 and assuming a normal distribution.
[d]One single point because the length of the interval for molting period is shorter than the length of the interval for the pre-refractory period.

curve. Values for these intervals (in days) are given in Table 5. Once again, stage 5 provides the least information, giving the smallest percentage of valid individuals for any value of the pre-refractory period (only up to 45% of individuals) (Fig. 5E) and the widest identified interval for the pre-refractory period (5.8–8.0 days) (Table 5). Table 5 also provides a summary of the estimated intervals for the pre-refractory, refractory and molting periods.

## Discussion

The findings of the present study show that, when the total insect weight is used as a threshold measure, the mean molting period is not significantly affected as the threshold value varies (the change is minimal in Fig. 3 for all stages; see the magenta coloured curve); this small variation implies a reasonable certainty with respect to the estimation of the mean molting periods for each instar. Recall that data are quite sparse, with a minimum of 2 days between feeding intervals. Therefore, note in Tables 1–3 (showing three different measures of threshold value) that the mean values for molting time for stages 1–4 are all well within the experimental data accuracy of 2 days. Data for stage 5 have more variation and the mean molting periods for the three measures are not within the experimental data accuracy of 2 days.

Because the results for weight, blood ingested and relative change in body weight in Tables 1–3 are all within experimental accuracy, we only use the absolute total body weight for the definition of the AUC measure, although any other measure could be used. The mean molting periods obtained from the AUC measure (Table 4) are shorter than those obtained from using only the total body weight measure (Table 1). We consider that all four measures used in the present study to estimate the threshold for molting are reliable despite the fact that the experimental setup does not permit the verification of the exact time that a triatomine would really begin piercing the mouse's dermis, nor the actual initiation of blood ingestion (J. E. Rabinovich, P. Medone & N. di Clemente, unpublished

data); our confidence is based on the fact that the dominant time invested (>90%) of a triatomine's complete meal is in the pumping process of blood to the anterior middle section of the intestine (Araújo *et al.*, 2012) and not in finding and accessing an adequate blood vessel.

Several individuals are removed from the study because they do not reject any offered meal. According to our methodology, we need the weight of the insect at the time when it rejects a meal (which is the latest time for the threshold to occur) for our calculations. To test the effect of removing such individuals (most of them correspond to long, 8- and 10-day, inter-feeding intervals), we approximate the weight of the insect in the interval from their last meal until molting. Our approximation is based on a linear weight decay with a slope the same as that for the insect's interval after its first meal. For those individuals with at least two meals taken, such an approximation is close to their observed weight. Having obtained the weight of the insect in its last interval before molting, we estimate threshold values and find them to be exactly the same (except for stage 1) as those computed before (when individuals were removed). Mean and variance of molting period are also very close to previous estimates. All of these results suggest that there is only a minimum effect on our results from the individuals removed from the original analysis.

With our experimental data, it is not possible to determine a critical weight as defined in Callier & Nijhout (2013). Not only weight, but also time is involved in triggering the molting process. This provides support for the use of AUC in the determination of the threshold for molting. However, our current AUC definition will not apply to very long intervals between meals because nymphs would eventually die if starved to an extremely low weight, which suggests that there must be a minimum weight and/or a maximum time before achieving the molting threshold for which nymphs can still survive and molt.

Although the refractory period (period from the moment a meal is first rejected until molting) could be estimated directly from the data, the pre-refractory period requires a clear definition of the measure used for determining the molting threshold because the definition of the pre-refractory period depends on the threshold itself. The use of the AUC allows a more accurate estimate of the pre-refractory period (Table 5). Although the precision of such estimate would be set by the shortest experimental time (2 days) when using total body weight as the threshold measure, the results based on the AUC suggest a pre-refractory period that is shorter than 2 days for some stages (Table 5). To the best of our knowledge there are no previous estimates of pre-refractory periods for *R. prolixus*, nor for any other triatomine species. Our estimates show a pre-refractory period in the range of 2 days for stages 1, 3 and 4, although it is much shorter (0–14 h) for stage 2, and much longer (5.8–8 days) for stage 5, which conforms well to the findings of Wigglesworth (1934). We do not have an explanation for the extremely short pre-refractory period of stage 2, which does not appear to be biologically sound.

Our confirmation of a weight-dependent mechanism as a threshold weight for molting could be related to the disinhibition of the secretion of the prothoracicotropic hormone (PTTH) and of the triggering of ecdysone by the Juvenile hormone (JH) (Knobloch & Steel, 1989). However, in this process, not only the necessary total body weight must be reached, but also this should happen at the right time. For example, despite the release of PTTH from the brain complex and the synthesis (and release) of ecdysteroids by the prothoracic glands both being confirmed in *R. prolixus*, a circadian rhythm also appears to be involved: Vafopoulou & Steel (2001) report that the prothoracic glands possess a circadian oscillator that is light sensitive *in vitro*, in addition to continuous light causing cessation of the release of PTTH.

The only study similar to our analysis in *R. prolixus* is that by Leis *et al.* (2016) who analyze the respiratory pattern and the energetic cost of taking a blood meal in *R. prolixus* and report an increase of up to 17-fold in the metabolic rate during feeding and a change in the respiratory pattern, which switches from discontinuous cyclic during resting to continuous when the insects start to feed, remaining in this unchanged condition for several hours. No differences are reported by Leis *et al.* (2016) between *R. prolixus* feeding on blood or on saline solution *in vitro*, revealing that the reason for such differences in the energetic cost is the substrate for feeding (vessels vs. membrane) and not the nature of the fluid. Leis *et al.* (2016) also report that *R. prolixus* water loss significantly increases during feeding, and that the mean respiratory quotient (RQ) of 0.83 in resting bugs decreases to 0.52 during feeding.

Although *M. sexta* larvae may not exclusively use the critical weight mechanism and may also use a size-independent mechanism that is also independent of the brain (Callier & Nijhout, 2011), an independence of the threshold weight mechanism is not observed in *R. prolixus*. Additionally, under starved conditions, triatomines do not attain molting, as is observed when *Drosophila melanogaster* larvae are starved (Beadle *et al.*, 1938; Stieper *et al.*, 2008); it is even suggested that starvation actually triggers the molt in *D. melanogaster* starved larvae, an option that possibly has to be discarded in the case of triatomines.

The calculation of the AUC is used in several contexts in the biological sciences, including endocrinological and pharmacological studies (Pruessner *et al.*, 2003; Drusano *et al.*, 2004). In most cases, the AUC is used to represent the accumulation of some quantity over a specific time period. For example, in pharmacokinetics, the average drug concentration (drug exposure) in an individual over a time interval is estimated as the AUC of drug concentration, with the corresponding units of (mass/volume) × time. In the present study, such a quantity is taken as the total body weight of *R. prolixus* at each instar, which we consider to be a novel methodology that may provide new insights with respect to the problem of determining the critical size/weight for molting in hemimetabolous insects, particularly because a time component in the molting process in *R. prolixus* is identified. Although the AUC indicator agreement with the data and a flexibility to estimate parameters appear to be appropriate, additional experiments (particularly with smaller feeding intervals than those of the present study) would provide further evidence of the applicability of this method based on the AUC concept on the molting process of *R. prolixus*.

From an epidemiological standpoint, the results of the present study may also contribute to the understanding of the spread of Chagas disease. Vectorial capacity of triatomines is higher when

insects defecate during or shortly after feeding and so infective faeces come in contact with the host, which is the source of transmission of parasites to the host (Trumper & Gorla, 1991). The results of the present study indicate that the pre-refractory period increases as the nymphal stages develop, particularly from stage 4 to stage 5 where there is an abrupt increase, suggesting that the larger stages should be more involved in Chagas disease transmission. Accordingly, the higher the pre-refractory period, the higher the risk of parasite transmission because insects feed more frequently (i.e. there is a larger contact rate between host and vector), thus increasing the probability of *T. cruzi* transmission. In other triatomine species, insects that feed repeatedly also defecate on hosts more often. Reisenman *et al.* (2011) report, in *Triatoma rubida*, that more than half of the immature insects exhibiting multiple feeding bouts (62%) are observed to defecate during interruptions of feeding, which would most likely occur on or near the host. Similar findings are obtained for *Rhodnius nasutus* (Oliveira *et al.*, 2009), *Rhodnius ecuadoriensis* (Villacis *et al.*, 2008), *Triatoma patagonica* and *Triatoma infestans* (Rodríguez *et al.*, 2008), *Triatoma vitticeps* (Biral dos Santos *et al.*, 2006), *Meccus pallidipennis* (Martínez-Ibarra & Novelo-López, 2004), *Triatoma rubrovaria* (Almeida *et al.*, 2003), *Triatoma rubrofasciata* (Braga & Lima, 1999) and *Triatoma barberi* (Zárate *et al.*, 1984). Therefore, the longer pre-refractory period that is observed as nymphal stages of *R. prolixus* develop suggests the need to focus vector control strategies on the latter stages, especially the last one.

From an evolutionary perspective, the results of the present study are in agreement with previous knowledge based upon the fasting capacity of triatomines. This insect group has evolved towards blood-feeding by developing several species-specific adaptations, including morphological, physiological and behavioural ones (mouthparts, saliva composition, enzymes and digestive symbionts, amongst others) (Otálora-Luna *et al.*, 2015). Therefore, a high fasting capacity and a high molting success might have been selected in triatomines under restricted conditions, such as interrupted feeding. In addition, the high molting rate (independent of the total amount of blood ingested) of *R. prolixus* that is observed in the present study is in agreement with individuals being well adapted to feed under unpredictable environmental conditions (unstable and uncertain host availability); thus, maximizing molting success could be considered as an evolutionary adaptive strategy of triatomines with respect to coping with such an environmental risk.

## Acknowledgements

We thank Marcelo Lorenzo, Gabriel Manrique and Pablo Guerenstein for reading a draft of this paper and providing very useful suggestions. A. Tongen would like to acknowledge funding from the Fulbright Scholars Program (Department of State) and the College of Science and Mathematics at James Madison University's educational leave program. The authors confirm that there are no disputes over the ownership of the data presented in this paper. All contributions have been attributed appropriately, via coauthorship or acknowledgement, as appropriate to the situation.

## References

Almeida, C.E., Francischetti, C.N., Pacheco, R.S. & Costa, J. (2003) *Triatoma rubrovaria* (Blanchard, 1843) (Hemiptera-Reduviidae-Triatominae) III: patterns of feeding, defecation and resistance to starvation. *Memórias do Instituto Oswaldo Cruz*, **98**, 367–371.

Araújo, R.N., de Figueiredo Gontijo, N., Lazzari, C.R. & Pereira, M.H. (2012) Interação entre os insetos hematófagos e seus hospedeiros vertebrados. *Tópicos Avançados em Entomologia Molecular* Capter 11 (ed. by M.A. da Silva Neto, C. Winter and C. Termignoni), pp. 1–15. Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Brazil.

Beadle, G.W., Tatum, E.L. & Clancy, C.W. (1938) Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*. *The Biological Bulletin*, **75**, 447–462.

Biral dos Santos, C., Rocha Leite, G., Sessa, P.A. & Falqueto, A. (2006) Dynamics of feeding and defecation in *Triatoma vitticeps* (Stal, 1859) (Hemiptera, Reduviidae, Triatominae) and its potential in the transmission of *Trypanosoma cruzi*. *Memórias do Instituto Oswaldo Cruz*, **101**, 543–546.

Braga, M.V. & Lima, M.M. (1999) Feeding and defecation patterns of nymphs of *Triatoma rubrofasciata* (De Geer, 1773) (Hemiptera: Reduviidae), and its potential role as vector for *Trypanosoma cruzi*. *Memórias do Instituto Oswaldo Cruz*, **94**, 127–129.

Callier, V. & Nijhout, H.F. (2011) Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 14664–14669.

Callier, V. & Nijhout, H.F. (2013) Body size determination in insects: a review and synthesis of size- and brain-dependent and independent mechanisms. *Biological Reviews*, **88**, 944–954.

Davidowitz, G., D'Amico, L.J. & Nijhout, H.F. (2003) Critical weight in the development of insect body size. *Evolution & Development*, **5**, 188–197.

Davidowitz, G., D'Amico, L.J. & Nijhout, H.F. (2004) The effects of environmental variation on a mechanism that controls insect body size. *Evolutionary Ecology Research*, **6**, 49–62.

Drusano, G.L., Preston, S.L., Fowler, C. *et al.* (2004) Relationship between fluoroquinolone area under the curve: minimum inhibitory concentration ratio and the probability of eradication of the infecting pathogen, in patients with nosocomial pneumonia. *The Journal of Infectious Diseases*, **189**, 1590–1597.

Ferreira, R.A., Lazzari, C.R., Lorenzo, M.G. & Pereira, M.H. (2007) Do haematophagous bugs assess skin surface temperature to detect blood vessels? *PLoS ONE*, **2**, e932.

Flores, G.B. & Lazzari, C.R. (1996) The role of the antennae in *Triatoma infestans*: orientation towards thermal sources. *Journal of Insect Physiology*, **42**, 433–440.

Fresquet, N. & Lazzari, C.R. (2011) Response to heat in *Rhodnius prolixus*: the role of the thermal background. *Journal of Insect Physiology*, **57**, 1446–1449.

Friend, W.G. & Smith, J.J.B. (1977) Factors affecting feeding by bloodsucking insects. *Annual Review of Entomology*, **22**, 309–331.

Hentschel, B.T. (1999) Complex life cycles in a variable environment: predicting when the timing of metamorphosis shifts from resource dependent to developmentally fixed. *American Naturalist*, **154**, 549–558.

Knobloch, C.A. & Steel, C.G.H. (1989) The timing of release of prothoracicotropic hormone in relation to the head critical period

during larval-adult development in *Rhodnius prolixus* (Hemiptera): evidence from head transplantation experiments. *Journal of Insect Physiology*, **35**, 459–464.

Lazzari, C.R. & Núñez, J.A. (1989) The response to radiant heat and the estimation of the temperature of distant sources in Triatoma infestans. *Journal of Insect Physiology*, **35**, 525–529.

Leis, M., Pereira, M.H., Casas, J. *et al.* (2016) Haematophagy is costly: respiratory patterns and metabolism during feeding in *Rhodnius prolixus*. *Journal of Experimental Biology*, **219**, 1820–1826.

Martínez-Ibarra, J.A. & Novelo-López, M. (2004) Blood meals to molt, feeding time and postfeeding defecation delay of *Meccus pallidipennis* (Stål, 1872) (Hemiptera: Reduviidae) under laboratory conditions. *Folia Entomológica Mexicana*, **43**, 313–319.

Nijhout, H.F. & Williams, C.M. (1974a) Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *Journal of Experimental Biology*, **61**, 481–491.

Nijhout, H.F. & Williams, C.M. (1974b) Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *Journal of Experimental Biology*, **61**, 493–501.

Nijhout, H.F., Davidowitz, G. & Roff, D.A. (2006) A quantitative analysis of the mechanism that controls body size in *Manduca sexta*. *Journal of Biology*, **5**, 16.

Oliveira, T.G., Carvalho-Costa, F.A., Sarquis, O. & Lima, M.M. (2009) Feeding and defecation patterns of *Rhodnius nasutus* (Hemiptera: Reduviidae), a triatomine native to an area endemic for Chagas disease in the state of Ceará, Brazil. *American Journal of Tropical Medicine and Hygiene*, **81**, 651–655.

Otálora-Luna, F., Pérez-Sánchez, A.J., Sandoval, C. & Aldana, E. (2015) Evolution of hematophagous habit in triatominae (Heteroptera: Reduviidae). *Revista Chilena de Historia Natural*, **88**, 1–13.

Pruessner, J.C., Kirschbaum, C., Meinlschmid, G. & Hellhammer, D.H. (2003) Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology*, **28**, 916–931.

Rabinovich, J., Leal, J.A. & Feliciangeli de Piñero, D. (1979) Domiciliary biting frequency and blood ingestion of the Chaga's disease vector Rhodnius prolixus Stahl (Hemiptera: Reduviidae), in Venezuela. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**, 272–283.

Reisenman, C.E., Gregory, T., Guerenstein, P.G. & Hildebrand, J.G. (2011) Feeding and defecation behavior of *Triatoma rubida* (Uhler, 1894) (Hemiptera: Reduviidae) under laboratory conditions, and its potential role as a vector of Chagas disease in Arizona, USA. *American Journal of Tropical Medicine and Hygiene*, **85**, 648–656.

Rodríguez, C.S., Carrizo, S.A. & Crocco, L.B. (2008) Comparison of feeding and defecation patterns between fifth-instar nymphs of *Triatoma patagonica* (Del Ponte, 1929) and *Triatoma infestans* (Klug, 1934) under laboratory conditions. *Revista da Sociedade Brasileira de Medicina Tropical*, **41**, 330–333.

Schilman, P.E. & Lazzari, C.R. (2004) Temperature preference in *Rhodnius prolixus*, effects and possible consequences. *Acta Tropica*, **90**, 115–122.

Schofield, C.J. (1994) *Triatominae: Biology and Control*. Eurocommunica Publications, U.K.

Stieper, B.C., Kupershtok, M., Driscoll, M.V. & Shingleton, A.W. (2008) Imaginal discs regulate developmental timing in *Drosophila melanogaster*. *Developmental Biology*, **321**, 18–26.

Trumper, E.V. & Gorla, D.E. (1991) Density-dependent timing of defaecation by *Triatoma infestans*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85**, 800–802.

Vafopoulou, X. & Steel, C.G.H. (2001) Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *Journal of Insect Physiology*, **47**, 935–941.

Villacis, A.G., Arcos-Teran, L. & Grijalva, M.J. (2008) Life cycle, feeding and defecation patterns of *Rhodnius ecuadoriensis* (Lent & Leon 1958) (Hemiptera: Reduviidae: Triatominae) under laboratory conditions. *Memórias do Instituto Oswaldo Cruz*, **103**, 690–695.

Vinauger, C., Pereira, M.H. & Lazzari, C.R. (2012) Learned host preference in a Chagas disease vector, *Rhodnius prolixus*. *Acta Tropica*, **122**, 24–28.

WHO (2016) *Chagas Disease (American trypanosomiasis). Fact Sheet* [WWW document]. URL http://www.who.int/mediacentre/factsheets/fs340/en/ [accessed on 16 October 2016].

Wigglesworth, V.B. (1934) Physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and metamorphosis. *Quarterly Journal of Microscopical Science*, **77**, 191–222.

Wigglesworth, V.B. & Gillett, J.D. (1934) The function of the antennae in *Rhodnius prolixus* (Hemiptera) and the mechanism of orientation to the host. *Journal of Experimental Biology*, **11**, 120–139.

Zárate, L.G., Morales López, G., Cabrera Ozuna, M. *et al.* (1984) The biology and behavior of *Triatoma barberi* (Hemiptera: Reduviidae) in Mexico IV. Feeding and defecation patterns. *Journal of Medical Entomology*, **21**, 548–560.

Accepted 25 April 2017