Co-existing locomotory activity and gene expression profiles in a kissing-bug vector of Chagas disease

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ABSTRACT

The triatomine bug *Rhodnius prolixus* is a main vector of Chagas disease, which affects several million people in Latin-America. These nocturnal insects spend most of their locomotory activity during the first hours of the scotophase searching for suitable hosts. In this study we used multivariate analysis to characterize spontaneous locomotory activity profiles presented by 5th instar nymphs. In addition, we investigated whether sex and the expression of the *foraging* (*Rpfor)* gene could modulate this behavioral trait. Hierarchical Clustering and Redundancy Analyses detected individuals with distinct locomotory profiles. In addition to a great variation in locomotory intensity, we found that a proportion of nymphs walked during unusual time intervals. Locomotory activity profiles were mostly affected by the cumulative activity expressed by the nymphs. These effects promoted by cumulative activity were in turn influenced by nymph sex. Sex and the *Rpfor* expression had a significant influence on the profiles, as well as in the levels of total activity. In conclusion, the locomotory profiles evinced by the multivariate analyses suggest the co-existence of different foraging strategies in bugs. Additionally, we report sex-specific effects on the locomotion patterns of 5th instar *R. prolixus*, which are apparently modulated by the differential expression of the *Rpfor* gene.

Keywords: *Rhodnius prolixus,* locomotory activity, gene expression, *foraging* gene, Chagas disease

1. INTRODUCTION

Locomotory activity is a complex behavioral trait which has been associated to diverse contexts such as foraging, the search for sexual partners or for appropriate hiding places (Franco et al., 2018; Guerenstein and Lazzari, 2009; Kaun and Sokolowski, 2009; Kohsaka et al., 2017; Takken and Knols, 1999). Insect locomotion can be modulated by diverse factors, as for example, genetic and phenotypic diversity and the way individuals interact in a community (Hughes et al., 2008), as well as age, sex, nutritional status, and circadian clocks (Cascallares et al., 2018; Pompanon et al., 1999). In addition, environmental conditions such as light, temperature and relative humidity will also impact insect locomotory activity profiles (Buchan and Sohal, 1981; Shou et al., 2013). Individual variation will be induced by these different endogenous or exogenous factors that affect the behavioral output through neural or neuroendocrine mechanisms (Zupanc and Lamprecht, 2000). In this scenario, the use of multivariate analyses to probe relations of parameters such as physiology, environment and development on locomotory patterns shown by animals becomes an interesting tool to address the intrinsic complexity hidden behind locomotory datasets (Ašmonaitè et al., 2016; Thorpe and Crompton 2006; Yamamoto et al., 2018).

Triatomines are hematophagous insects, which in addition to taking blood from vertebrates, act as vectors of *Trypanosoma cruzi.* This parasite is the etiological agent of Chagas disease, a neglected health condition that currently affects 5-7 million people, particularly in Latin America (WHO, 2017). These nocturnal insects remain hidden in protected shelters during the day as a consequence of their intense thigmotaxis and negative phototaxis (Mota and Lorenzo, 2012; Reisenman et al., 1998; Ward and Finlayson, 1982). During dusk, with the decrease in light intensity, triatomines eventually start a period of spontaneous locomotion, leaving their shelters to search for hosts and then, a few hours before and during dawn they return to the safety of the refuge (Ferreira et al., 2019; Lorenzo and Lazzari, 1998). The intensity of the activity of triatomines depends on factors such as nutritional status (Bodin et

al., 2008; Guarneri et al., 2003; Lorenzo and Lazzari, 1998) and maturation (Bodin et al., 2009). The patterns of this spontaneous locomotory activity, as well as their regulation by endogenous clocks, were well characterized in *Triatoma infestans* (Lazzari, 1992). The daily patterns of locomotory activity of triatomines exhibit a bimodal profile, with an initial peak during the first hours of the scotophase and a second and narrower one that starts preceding the photophase (Guarneri et al., 2003; Lazzari, 1992; Lorenzo and Lazzari, 1998; Marliére et al., 2015; Pavan et al., 2016). Previous studies on triatomine locomotory activity have focused their analyses on average tendencies. We suggest here that the factors underlying the inherent variability of triatomine locomotion deserve attention, as they may reveal relevant biological features affecting bug performance under different predation risk scenarios.

Foraging (*for*) is an essential gene with pleiotropic effects on development and behavior (Hofmann, 2005). It encodes a cGMP-dependent protein kinase (PKG) that is found in diverse groups of organisms from *Paramecia* to humans and presents very conserved functions (Feil et al., 2005). In invertebrates, the *foraging* gene has been associated with the modulation of food-related behavior in different groups such as *Drosophila* (de Belle et al., 1989; Osborne et al., 1997; Pereira and Sokolowski, 1993), honey bees (Ben-Shahar, 2005), ants (Ingram et al., 2005), nematodes (Fujiwara et al., 2002), social wasps (Tobback et al., 2008) and locusts (Luccas et al., 2010). We have previously shown that the levels of locomotory activity of *Rhodnius prolixus* are altered by trypanosome infection (Marliére et al., 2015). These changes coincided with an altered expression of *Rpfor*, the *foraging* gene orthologue of this species, suggesting that it participates in the modulation of triatomine activity.

In the present study, we characterized the distribution of locomotory events of starved 5th instar nymphs of *R. prolixus* along a 24h interval. Then, we tested whether bug locomotory profiles would clusterize evincing differing activity profiles. We subsequently hypothesized that differing locomotory profiles correlate with *Rpfor*

expression. Furthermore, we decided to explore whether nymph sex would affect bug locomotory profiles as seen for adult *R. prolixus* (Pontes et al., 2014). Finally, we addressed whether these variables also affected the cumulative locomotion observed for each individual.

2. METHODS

2.1 Insects

Rhodnius prolixus specimens used in our study came from a colony derived from insects collected in Honduras around 1990. Insects were maintained by the Vector Behavior and Pathogen Interaction Group at René Rachou Institute, Belo Horizonte, Brazil. Experimental bugs were fed citrated rabbit blood obtained from CECAL (Fiocruz, Rio de Janeiro, Brazil) offered through an artificial feeder at 37°C, alternating with blood from anesthetized chicken. The latter were anesthetized with intraperitoneal injections of a mixture of ketamine (20 mg/kg; Cristália, Brazil) and detomidine (0.3 mg/kg; Syntec, Brazil), under license number LW-61/2012 (Committee for Ethics in the Use of Animals, CEUA-FIOCRUZ).

2.2 Locomotory activity

The locomotory activity of nymphs was evaluated by means of an automatic actometric system previously developed by our group (for details see Marliére et al., 2015). Briefly, the device consists of 40 individual recording units (5 x 10 x 2 cm boxes), each featuring three infrared (IR) light emitting diodes (LEDs) positioned face to face with their respective receivers to detect insect movement. This device was used to record bug locomotory activity in all assays reported in this study. During the experiments, room temperature was kept at 27$\pm$1°C and illumination provided at 12:12 LL/DD (light intensity = 60 LUX). Fifth instar nymphs starved for 30 days after ecdysis were used in all experiments to grant an adequate bug motivation for the expression of spontaneous host search activity (Ferreira et al., 2019). In each assay, nymphs were individually placed in the recording units that contained a piece of filter

paper as a substrate lining the floor of each box (5 x 10 cm). Once an assay was set, the number of movement events was continually recorded for each individual.

A preliminary experiment in which 32 nymphs were released and their movements recorded for 72 hours, allowed comparing the number of movement events recorded for each of the three daily intervals. The corresponding dataset showed that the number of movements over the recorded interval showed less variability after the first day (data shown in Supporting Information Figure S1). As a consequence we chose to restrict subsequent data sampling to the second day.

Two experiments were performed to analyze whether different locomotory profiles could co-exist in our bug colony. A first scrutiny characterized the hourly activity profile of 548 nymphs through a 24h interval. In a second experiment, we selected a subsample of 65 nymphs reflecting the activity level diversity of the previous bug sample and sorted them by sex (Espínola, 1966). Afterwards, these insects were used to quantify the relative expression of the *Rpfor* gene by qPCR (RTq-PCR). In total, 32 males and 33 females were used for this second experiment.

2.3 RNA extraction and cDNA synthesis

We initially evaluated the expression of the *Rpfor* gene in samples made of pooled brains or fat bodies obtained from insects with different physiological status. Gene expression patterns reflected physiological changes similarly for both tissues (Supplementary Figure S2). Based on this, subsequent qPCR experiments focused on fat bodies, because samples from single individuals provided enough RNA for qPCR experiments. Fat body samples were dissected between 14-18h after the end of locomotory activity assays. Total RNA was extracted using TRIzol™ (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions. The total RNA obtained was treated with Turbo DNA-free kit (Invitrogen, Vilnius, Lithuania) to eliminate genomic DNA. Sample concentration was determined using a Qubit 2.0 Fluorometer (Life Technologies, Eugene, Oregon, USA). cDNA was synthesized from 500ng of mRNA using the M-MLV Reverse Transcriptase enzyme (Promega, Fitchburg, WI, USA) and a modified oligo dT primer (MgdT

5'-CGGGCAGTGAGCAACG (T12)-3') (Ursic-Bedoya et al., 2008) under the following conditions: 37°C for 60min and 95°C for 5min.

2.4 Quantification of *Rpfor* gene expression

Specific primers for *Rpfor* (Supplementary Table S1) were used in qPCR reactions to evaluate gene expression levels in individuals showing different locomotory profiles. All reactions contained 1µl of cDNA, 0.5µl of each primer (10µM), and 5µl of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen, Carlsbad, California, USA) in a final volume of 10µl. Experiments were conducted at the qReal-Time PCR Facility – RPT09D PDTIS/René Rachou Institute/FIOCRUZ MG in a ViiA 7 System (Applied Biosystems, Foster City, California, USA) under the following conditions: 10min at 96°C, followed by 40 cycles of 15sec at 95°C and 20sec at 60°C. Melting curve analyses confirmed the specificity of the reaction. All individual cDNA samples were evaluated in two technical replicates. qPCR results were analyzed by calculating the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Relative levels of expression for each insect were compared using α-tubulin (TUB) as a reference gene (Paim et al., 2012) (Supporting Information Table S1).

2.5 Statistical analysis

2.5.1 Hierarchical clustering and multivariate analyses

Two data matrices were built based on the activity profiles of individual nymphs. The first dataset encompassed the recordings obtained with 548 individuals throughout the 24h interval. The second dataset included the number of movement events and molecular data obtained with the sex-sorted nymphs. Data analyses were performed using the R-software (v3.4) on the Rstudio suite version 1.1.423 (Team, 2015). A hierarchical clustering dendrogram and its corresponding heatmap were generated using the heatmap3 package (v1.1.1). As we considered the absence of movement (joint absences) to be biologically informative, we opted to follow the workflow suggested by Anderson et al. (2011). This implied estimating the pairwise

dissimilarities between the objects in the matrix based on non-transformed raw counts and Euclidean indices. For this purpose, the 'vegdist' function from the R-package 'vegan' (v2.5.2) was called within the heatmap3 function script.

To address the effect of potentially relevant biological variables accountable for the variance within the matrices, multivariate ordination tests of the RDA type (redundancy analysis based on an ordinary unweighted linear regression, and unweighted singular variable decompositions) were executed considering their respective explanatory matrices (dummy matrices). Briefly, for each of the evaluated datasets, the dummy matrices contained explanatory data such as sex [male/female] (for 65 nymphs); total locomotion counts during the 24h span; and normalized gene expression coefficients (for 65 nymphs). The analyses and corresponding biplots (scaling=2, correlation plots focusing on response variables) were generated using the 'rda' function from the 'vegan' package (v2.4.6). Finally, to assess whether the hypothesized constraints could significantly explain the variance within the datasets, and thus how the response and explanatory variables interact, permutation-based MANOVA tests (PERMANOVA) were performed using the 'adonis2' function within the 'vegan' package (same version aforementioned) executing 9,999 permutations (Anderson, 2001; Anderson and Walsh, 2013). This multivariate analysis also generates an effect-size estimate (R^2) of the variance proportion explained by each of the hypothesized explanatory variables. A similar hypothesis testing approach was executed by Liu et al. (2015), when addressing how multiple biological effectors might contribute differently to the activity measured for zebrafish larvae.

2.5.2 *Rpfor* gene expression: its correlation with sex and locomotor activity

Data depicting expression levels for the *Rpfor* gene in male and female nymphs were tested for normality using the Shapiro-Wilk test. As expression levels did not adjust to a normal distribution, results were compared by means of the Mann Whitney test. A GLM-ANOVA was performed to test whether *Rpfor* expression and sex would influence the cumulative locomotory activity (24h). Then, the Spearman's correlation

coefficient was used to examine whether the cumulative activity was correlated to the relative expression of *Rpfor* gene for each sex separately.

3. RESULTS

The median number of locomotion events recorded for the 548 nymphs during the 24h interval was of 246.5 (0-2,753). Figure 1 presents the activity profile obtained showing the characteristic two peaks described by previous reports studying triatomine bugs (Guarneri et al., 2003; Lazzari, 1992; Lorenzo and Lazzari, 1998; Marliére et al., 2015; Pavan et al., 2016). These peak hours (8 and 20h) concentrated approximately 18 and 16% of the movement events, respectively. In general, a higher activity was observed during the first four hours of the scotophase, which represented approximately half of all locomotory activity expressed. However, as indicated by the amplitude between min and max values, the number of movement events recorded varied expressively (Figure 1).

To search for patterns based on individual locomotory activity profiles, we applied a multivariate analysis to generate a pairwise Euclidean distance matrix among hourly locomotion counts during the 24h interval. A hierarchical clustering test was executed based on the recordings of individual activity (i.e., number of locomotion events *per* individual *per* hour). As seen in Figure 2, three major blocks encompassing individuals with resembling dissimilarity patterns were identified based on dendrogram topology, heatmap intensity, and activity scatter plot (referred to as blocks 1, 2 and 3). Block number 1 (blue) contained 168 insects presenting an average of 461 movement events (113-1,468), while the 56 individuals from block 2 (green) showed an average of 918 events (410-2,753), and those in block 3 (pink, including 324 insects) an average of 147 events (0-796). Furthermore, by subdividing block 3 along its first node this cluster split in groups A (149) and B (175 individuals), showing an average of 251.2 (82-796) and 59.5 events (0-299), respectively. Of the total set of individuals, 30.7% belonged to block 1, 10.2% to block 2 and 59.1% to

block 3 (subgroup A 27.2 and B 31.9%). The most active bugs were assigned to block 2, while the least active ones grouped in block 3B, which included those that remained inactive through the assay.

The RDA analysis using cumulative individual activity (i.e., the total number of locomotion events *per* individual over 24h, henceforth referred to as total activity) as constraining variable, accounted for 42.59% of the total variance within the axes shown in Figure 3 (RDA1; and PC1-summarizing unconstrained variance). From that, 29.27% of the variation was due to the proposed explanatory variable (total activity), and its influence upon the objects and their correlations is represented along axis RDA1. The length of the vector is a measure of the magnitude of the response variable (total activity). The dispersion observed between individuals points to the existence of different activity profiles. Insects with low or null activity were positioned at the base of the total activity vector. Thirty-seven nymphs (6.7%) that presented zero locomotion counts for the whole 24h interval evaluated clustered at an ≈180° angle regarding the total activity vector (scores converging approximately around the [-0.4,4.8e-2] coordinates). As the number of movement events increased, bugs were distributed in the two dimensions following a bimodal pattern in which individuals with similar total activity counts (but different temporal profiles) separated along the ordinate space (examples of individual *per*-hour activity profiles are shown in Supplementary Figure S3). The further they followed the explanatory vector, the higher the magnitude of their response to this constraint, and their dissimilarity with the other individuals. The hourly intervals showing a stronger effect on total locomotion were 20, 21, 22, 23, and 24 (grouped below the activity vector). The hourly intervals corresponding to the photophase (from 9 to 19) grouped in the upper right quadrant of the ordinate space. The hourly intervals preceding the beginning of the photophase grouped close to the activity vector indicating that they share similar characteristics. In fact, this interval (except for 8h) presented low and uniform locomotory activity for all nymphs.

Searching for factors potentially related to the locomotory profiles, a new RDA was performed based on the hourly activity of 65 *R. prolixus* nymphs over 24h, and considering their total activity, sex and *Rpfor* gene expression as explanatory variables (Figure 4). According to the RDA, an estimated 45.32% of the variance among the profiles could be explained by the three variables. Total activity was the variable with the largest vector magnitude (Figure 4). The position of the vector representing *Rpfor* expression (≈-270°) suggests a negative relation with the activity vector (Figure 4). Males and females spread out differently along the activity vector, with the former dispersing preferentially through the space above the activity vector and the latter through the lower space. The position of the geometric means (centroids) in relation to the activity vector suggests different profiles of locomotory activity for males and females. We tested this hypothesis of sexually different locomotory profiles in a third dataset (including the hourly locomotion profiles of 175 individuals shown as Supplementary Figure S4) and obtained a similar outcome. We therefore compared the levels of *Rpfor* expression in males and females and found that *Rpfor* gene expression was significantly higher for female nymphs (Supplementary Figure S5, Mann Whitney, p=0.019).

A PERMANOVA test was then performed to address the significance with which each of the proposed variables explained the response matrix variations (Table 1). Globally, each of the variables tested, and the interaction between them drove 66.29% of the variance encompassed in the response matrix. Total locomotory activity was the most significant explanatory variable affecting locomotion profiles, presenting the largest effect size (Table 1; PERMANOVA, p=0.001). Also significant, but with smaller effect sizes, were sex, *Rpfor* expression and the interaction between sex and total activity (Table 1; PERMANOVA, p=0.004, p=0.005 and p=0.01, respectively). The significant interaction between sex and total activity indicated that the effects of total activity on the locomotion profiles were influenced by nymph sex.

As bug cumulative activity was the main factor influencing the different locomotion profiles, we tested whether the levels of activity were affected by gene expression and sex. We found a significant effect of sex and *Rpfor* expression on total bug activity (Table 2; GLM-ANOVA, $p<0.0001$ for both parameters). The significant interaction between sex and *Rpfor* expression (Table 2; GLM-ANOVA, $p<0.0001$) indicated that the effects of *Rpfor* expression on the levels of total activity were highly affected by nymph sex. We further tested whether total activity and gene expression were correlated in each of the sexes. A positive, but non-significant, correlation between total activity and *Rpfor* expression was found for female nymphs (Spearman correlation, $rS=+0.32$; $p=0.067$). Interestingly, a significant negative correlation between total male activity and *Rpfor* expression was detected (Spearman correlation, $rS=-0.67$; $p>0.0001$).

4. DISCUSSION

As reported in the literature (Guarneri et al., 2003; Lazzari, 1992; Lorenzo and Lazzari, 1998; Marliére et al., 2015), the insects tested in this study showed two peaks of activity in both, the beginning of the scotophase and photophase. As expected, half of all locomotion events were concentrated in the first hours of the scotophase. Nevertheless, the pairwise comparison of hourly movement events uncovered clearly distinct activity profiles. The hierarchical clustering divided our locomotion dataset into three principal branches as a result of the activity levels recorded and the hour in which movement was detected. Interestingly, more active insects showed the highest dissimilarities, while insects that tended to stay motionless showed higher homogeneity. Our results suggest that different locomotory strategies co-exist in *R. prolixus*. We acknowledge the intrinsic limitations of laboratory colonies in terms of genetic variability. Therefore, it would be relevant to test whether the locomotion profiles of sylvatic *R. prolixus* resemble those reported here.

As expected, the multivariate analyses (HC and RDA) suggested that the temporal dimension was the main factor influencing activity levels. Interestingly, two unknown features of kissing-bug locomotory patterns were revealed by applying these analyses. Firstly, we determined that our robust bug sample consists of individuals showing differentiated levels of activity, as shown in the three main branches created by the dendrogram. At the extremes, approximately 7% of the nymphs did not move, while 3% of them produced more than a thousand movement events during the same interval. The very different activity profiles reported in the dendogram may represent coexisting energy investment strategies and their ecological relevance deserves to be studied. Nutritional shortage scenarios are common in the sylvatic ecotopes of triatomines (Noireau and Dujardin, 2001; Sarquis et al., 2010). Under such circumstances, low activity could be advantageous to avoid consuming nutritional reserves while waiting for a blood-source to arrive to the ecotope. As an alternative strategy, intense locomotion could allow other individuals to approach a distant host, overcoming their nutritional shortage at increased predation risks induced by exposure outside shelters.

The second uncovered feature was the presence of individuals with similar activity levels that grouped separately according to dissimilarities in their temporal profiles. More specifically, their main activity bouts were expressed at different times. In order to explore the dataset in more detail we decided to focus on the expression of activity during the photophase. To avoid considering activity belonging to the tails of the main peaks, we excluded the two hours after lights on and before lights off from this calculation. This revealed that 21.9% of the insects were active in half of the hours belonging to this interval. Furthermore, 1.8% of them moved in all hours of the photophase. This temporal locomotory spread may also be an advantageous strategy in a nutritional shortage scenario, as foraging outside conventional hours would allow feeding on nocturnal vertebrates when these enter their resting state. In fact, opossums are fundamentally nocturnal and their blood is consistently detected in the gut of sylvatic insects (Hernández et al., 2016; Sasaki et al., 2003). It is important to acknowledge that this was not the predominant locomotion profile,

suggesting that being active during daytime increases predation risk. It is also worth pointing out that this experiment was performed with individuals that lacked access to shelters and co-specific aggregations. According to our current knowledge about shelter use by kissing-bugs, most individuals tend to group inside a refuge each night and only a small fraction of them leaves the protected places spontaneously (Ferreira et al., 2019; Lorenzo and Lazzari, 1998). In this sense, future studies are necessary to address whether insects that leave shelters in the absence of host cues belong to any of the different activity profiles seen here. To confirm the existence of individuals which are active during the day, one should also evaluate the dynamics of the use of shelters during the light phase, in both the presence and absence of host cues.

Our results are also suggestive of sex-specific behavioral profiles expressed by *R. prolixus* 5th instar nymphs. Sexually dimorphic locomotion patterns have been reported previously for house flies and *Drosophila melanogaster* (Bahrndorff et al., 2012; Belgacem and Martin, 2002; Buchan and Sohal, 1981; Helfrich-Förster, 2000; Martin et al., 1999; Ragland and Sohal, 1973). This is also the case for triatomines, for which adult bug behavior differs according to sex, as male kissing-bugs show active locomotory responses to sexual signals, while females do not (Manrique and Lorenzo, 2012). Indeed, the motivation to leave shelters is strikingly different for male and female kissing-bugs (Pontes et al., 2014). These authors showed that a relevant proportion of adult female bugs leaves shelters spontaneously and forages in the absence of host cues. Notably, the presence of males in the same environment does not alter this profile (Pontes et al., 2014). On the other hand, female odors promote the activation and orientation of adult males that otherwise do not seem to leave shelters. Ours is the first study reporting sex-specific locomotory activity profiles in immature triatomines. As mentioned, bugs were not exposed to shelter-associated stimuli or co-specifics, which would induce akinesis and aggregation behavior. Further studies should clarify whether sex-specific differences in locomotory activity expressed by nymphs relate to the search for sexual signals by immature male insects.

The expression of *Rpfor* influenced spontaneous locomotory activity significantly, reinforcing its suggested role in the modulation of foraging profiles. In addition, we found that the *Rpfor* gene affects total bug activity in a sex-dependent way. In fact, the inverse correlation between the total activity vector of males and *Rpfor* expression, suggests that decreased *Rpfor* expression induces increased male locomotory activity. Positive (Ben-Shahar et al., 2002; Lucas et al., 2010; Pereira and Sokolowski, 1993; Tobback et al., 2011) and inverse (Ingram et al., 2005, George et al., 2018; Tobback et al., 2008) correlations between *foraging* expression and locomotory activity have been reported.

In conclusion, cluster analysis evinced distinguishable locomotion patterns that may represent diverse energy investment strategies. Then, multivariate analyses have provided original perspectives on the molecular control of the expression of kissing-bug locomotory activity. Furthermore, they allowed us to identify unexpected sex-specific patterns of 5th instar nymph locomotion, which are apparently affected by the differential expression of the *Rpfor* gene.

Competing interests

No competing interests declared.

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Tables and Figures

Table 1. Permutational non-parametric multivariate analysis of variance.

Factor	DF	R^2	F	Pr(>F)	
Total_activity	1	0.5455	93.842	0.001	**
Sex	1	0.0294	5.059	0.004	**
Rpfor	1	0.0424	7.305	0.005	**
Sex:*Rpfor*	1	0.0083	1.439	0.200	
Sex:Total_activity	1	0.0287	4.948	0.011	*
Rpfor:Total_activity	1	0.0083	1.427	0.215	
Residual	58	0.3371			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Evaluated formula = matrix65v24 ~ (((Sex * FOR)) + (Sex * Total_activity) + (FOR * Total_activity)), data = dummy65iexp, permutations = 999, method = "euclid", by = "terms").

Table 2. GLM analysis of the effects of *Rpfor* expression and sex on the cumulative activity of *Rhodnius prolixus* nymphs.

	Estimate	Std. Error	Z Value	Pr(>z)	
(Intercept)	5.4849	0.0217	252.60	<2e-16	***
Sex	1.8700	0.0279	66.95	<2e-16	***
Rpfor	1.4693	0.0676	21.73	<2e-16	***
Sex:*Rpfor*	-7.3204	0.1237	-59.16	<2e-16	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.

Evaluated formula = glm(formula = Total_activity ~ Sex * FOR, family = poisson())

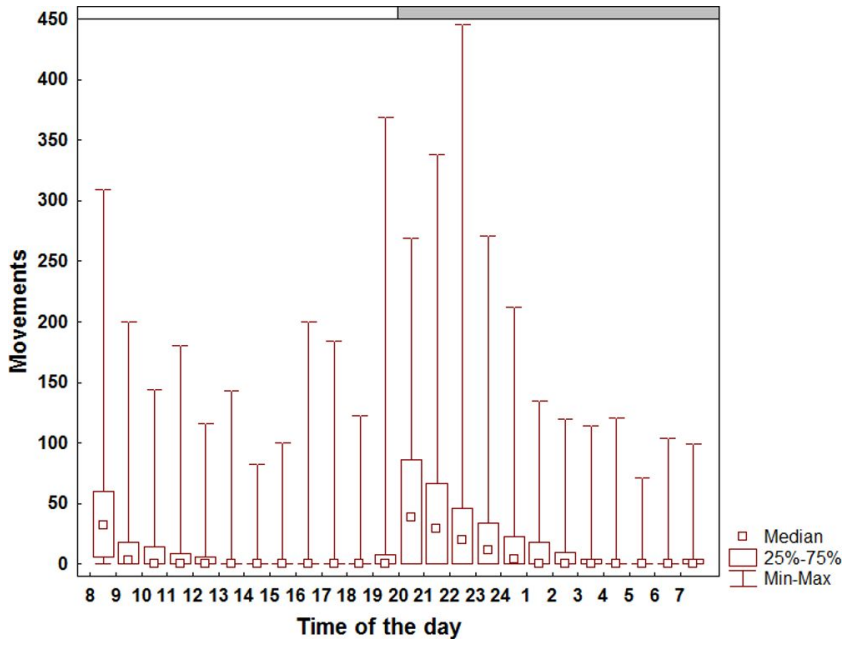


Figure 1. Hourly median of movement events shown by 5th instar *Rhodnius prolixus* nymphs over a 24h interval. The white and gray bars represent the photophase and scotophase, respectively. Data are presented as the median, 25%-75% quartiles, minimal and maximal values of the number of movement events *per* hour based on data collected with 548 insects.

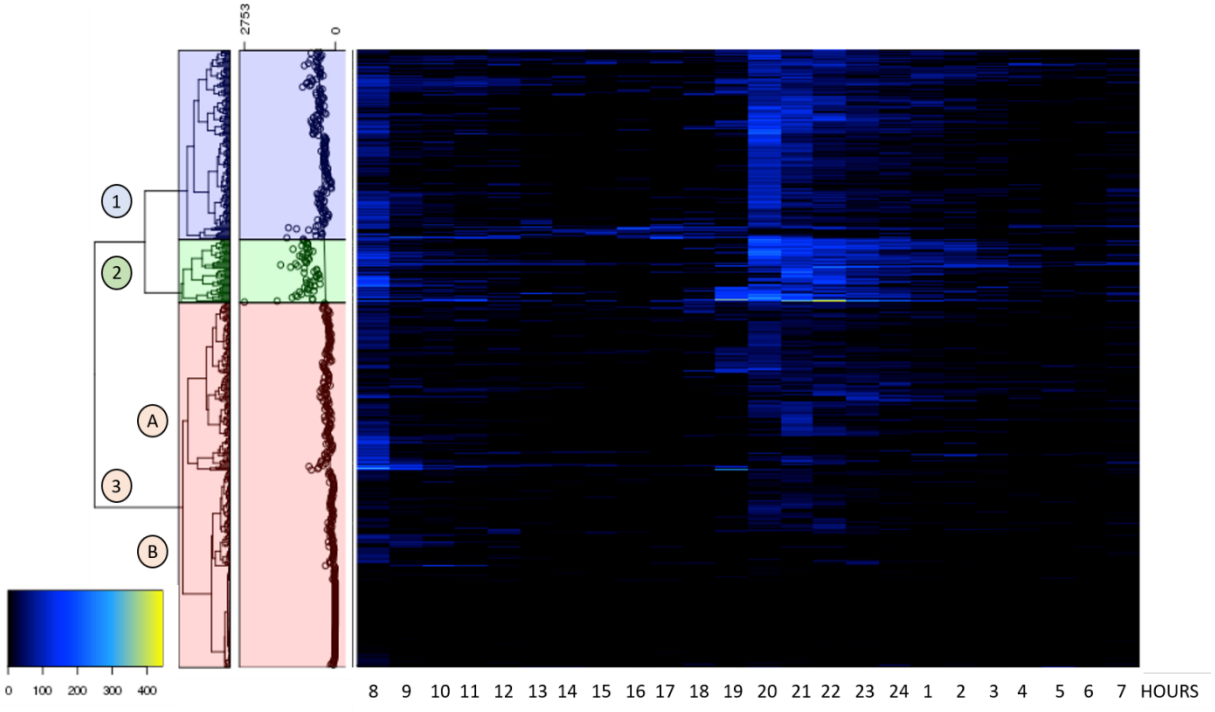


Figure 2. Hierarchical clustering of triatomine locomotory profiles based upon number of movements *per* hour and their pairwise Euclidean distances. The heatmap depicts the degree of dissimilarity amongst the 548 individuals during 24h. The middle panel, between the heatmap and the dendrogram, presents a scatter plot of the total activity recorded for each individual throughout the selected interval. The colored areas and trend line in the scatter plot aid visualizing the dendrogram topology as they discriminate deeper node tips from each other according to the proposed blocks based on total locomotion counts and dissimilarity patterns. Block number 1 (blue) contains 168 insects, block 2 (green) is composed by 56 individuals, and block 3 (pink) by 324 insects. Block 3 was subdivided along its first node in groups A and B, with 149 and 175 individuals, respectively).

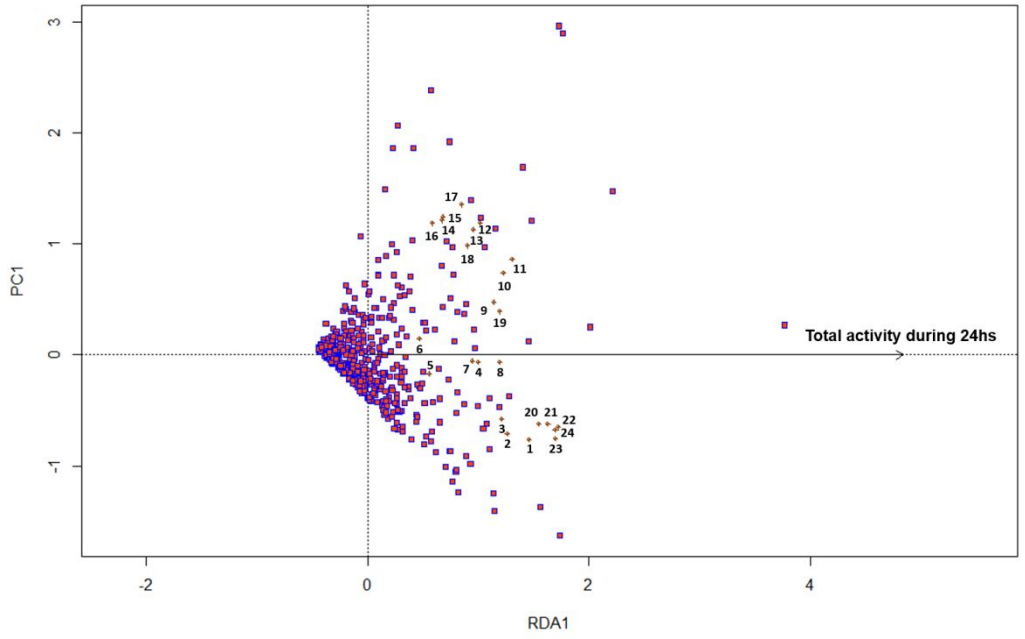
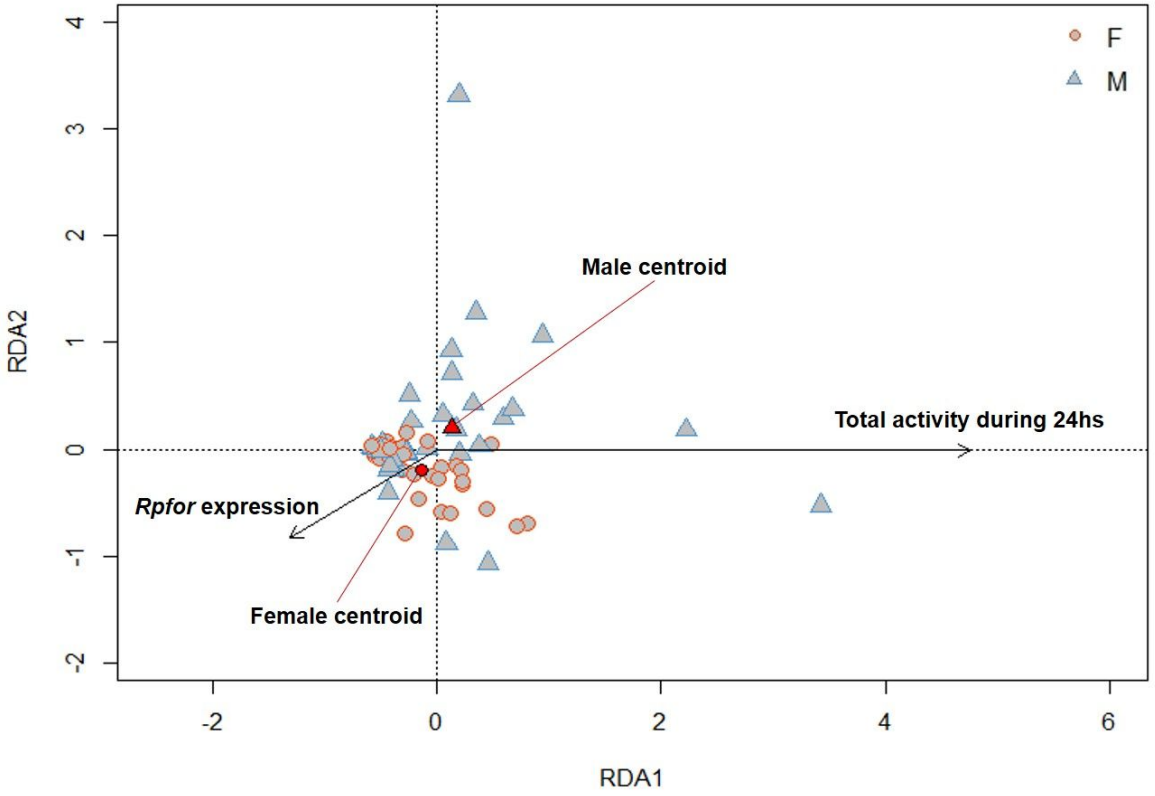


Figure 3. Redundancy analysis (RDA) biplot considering total activity throughout a 24h interval as explanatory variable. The RDA depicts how individuals dispersed on the (constrained) ordination space based upon Euclidean dissimilarities between their locomotion profiles. The patterns of variation within the dataset of response variable measures were constrained by the total activity counts recorded during a 24h interval (vector pointing right along the horizontal axis). In total, 42.59% of the variance is depicted in the biplot. From it, the proposed explanatory variable (total

activity) accounts for 29.27% of the total variance within the response matrix and is depicted as axis RDA1. Axis PC1 encompassed 13.32% of the unconstrained variance. Each square represents one individual in the ordinate space. The numbers represent the hours of the day.



RDA1: Encompasses 98.09% out of the 45.32% variance constrained by the proposed variables.
RDA2: Encompasses 1.52% out of the 45.32% variance constrained by the proposed variables.

Figure 4. Redundancy analysis (RDA) biplot considering biological effectors. The RDA plot depicts how male and female individuals distributed along the biplot. Locomotion profile dissimilarities were constrained by explanatory variables accounting for total activity during 24h, sex, and *Rpfor* gene expression profiles. In total, 45.32% of the variance within the matrix is driven by the proposed variables. From it, 98.09% of the constrained variance resolved along the horizontal axis (RDA1), whereas 1.52% is resolved along RDA2. Male and female locomotion profiles were found to differ significantly based on the permutational MANOVA.