Rhodnius prolixus and *Rhodnius robustus*–like (Hemiptera, Reduviidae) wing asymmetry under controlled conditions of population density and feeding frequency

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Habitat change in *Rhodnius* spp may represent an environmental challenge for the development of the species, particularly when feeding frequency and population density vary in nature. To estimate the effect of these variables in stability on development, the degree of directional asymmetry (DA) and fluctuating asymmetry (FA) in the wing size and shape of *R. prolixus* and *R. robustus*–like were measured under laboratory controlled conditions. DA and FA in wing size and shape were significant in both species, but their variation patterns showed both inter-specific and sexual dimorphic differences in FA of wing size and shape induced by nutrition stress. These results suggest different abilities of the genotypes and sexes of two sylvatic and domestic genotypes of *Rhodnius* to buffer these stress conditions. However, both species showed non-significant differences in the levels of FA between treatments that simulated sylvan vs domestic conditions, indicating that the developmental noise did not explain the variation in wing size and shape found in previous studies. Thus, this result confirm that the variation in wing size and shape in response to treatments constitute a plastic response of these genotypes to population density and feeding frequency.

1. Introduction

In general, in bilateral species, symmetric characters are expressed identically on both the left and the right sides of the body, given that morphological characters on each side are controlled by the same genotype (Palmer 1996). However, deviations from perfect symmetry are often observed in many species and they have been interpreted as indicators of developmental instability (Palmer 1996). One way to measure developmental instability is by using fluctuating asymmetry (FA), a measure of small, random and non-directional deviations between the left and the right values of a symmetrical morphological character (Van Valen 1962).

FA is often inferred as an indicator of the degree of perturbation when invertebrate or vertebrate taxa are under stressful conditions, as in the case of insecticide use (Hardersen 2000; Hoffmann *et al.* 2005; Ondo Zue Abaga *et al.* 2011), inbreeding (Réale and Roff 2003; Carter *et al.* 2009) or nutritional stress (Breuker and Brakefield 2003; Stige *et al.* 2004; Soto *et al.* 2008). FA has also been used to monitor environmental pollution with potential effect on stress and fitness reduction (Lens *et al.* 2002; Leamy and Klingenberg 2005), to compare conspecific populations inhabiting different geographic areas (Kark 2001) or exposed to different ecological conditions (Nicieza *et al.* 2006; Cuervo and Restrepo 2007); and to analyse developmental noises (Gibbs and Breuker 2006).

The studies on the effect of nutritional stress during development on FA have produced contradictory results. Some studies in birds have shown that FA increases under nutritional stress (Swaddle and Witter 1994; Pravosudov and

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Kitayski 2006; Sillampaa *et al.* 2010), while other studies addressed in species of different taxa showed that FA remained unaffected (Bjorksten *et al.* 2000; Gronkjaer and Sand 2003; Searcy *et al.* 2004; Stige *et al.* 2004; Vijendravarma *et al.* 2011). For example, in *Drosophila* spp contradictory results have been obtained in several bilateral traits under nutritional stress (Imasheva *et al.* 1999; Woods *et al.* 2002; Vishalakshi and Singh 2009; Vijendravarma *et al.* 2011). Increased levels of FA were found in three bilateral traits (wing length, sternopleural chaeta number and arista branch number) in adults of *D. melanogaster* reared on low yeast-sugar diet (Imasheva *et al.* 1999). However, unaffected levels of FA were also found in five bilateral traits (wing length, sternopleural bristle number, wing–thorax ratio, ovariole number in females and sex comb teeth number in males) in *D. melanogaster* reared on low-yeast and high-ethanol conditions (Woods *et al.* 2002). Similarly, unaffected levels of FA were found in *D. ananassae* reared on poor yeast food, although this species showed high levels of FA under larval crowding, which produces both nutritional stress and high exposition to toxic waste products (Vishalakshi and Singh 2009).

The effect of nutritional stress on FA has not yet been addressed in Triatominae, a subfamily of Reduviidae (Hemiptera, Heteroptera), which includes 141 species of haematophagous insects taxonomically arranged in 5 tribes and 15 genera (Schofield and Galvao 2009; Jurberg *et al.* 2009). Most of these species are capable of transmitting the protozoan parasite *Trypanosoma cruzi* – causative agent of Chagas disease – although relatively few are of epidemiological significance as vectors of *T. cruzi* to humans (Schofield and Galvao 2009). In fact, the Triatominae species of greatest epidemiological importance have domestic habitats since they have adapted to live in close contact with humans, although these are currently subject to a series of large-scale control interventions (Schofield *et al.* 2006). On the other hand, the sylvan species are not associated with humans and sometimes are distributed in sympatry with the domestic species (Feliciangeli *et al.* 2007; Fitzpatrick *et al.* 2008).

Given that the domestication of Triatominae can be envisaged as a successful invasion from the sylvan to the domiciliary habitat (Schofield *et al.* 1999), the species *Rhodnius prolixus* and *R. robustus*–like provide a good model system to study the evolutionary and ecological bases of morphological variation, as well as fluctuating asymmetry. These two phylogenetically related species of *Rhodnius* exhibit different abilities to colonize domestic and sylvan habitats. They belong to the 'robustus' group, the major lineage of the tribe Rhodniini of Triatominae, which comprises members with principally cis-Andean distribution in South America (Abad-Franch and Monteiro 2007). *R. prolixus* is considered the primary vector of Chagas disease in Colombia, Venezuela, and some countries of Central America (Dias *et al.* 2002) because it is essentially a domestic species. However, it has

been found occupying the same sylvan habitat with its closely related and morphologically similar species *R. robustus*, which has a minor epidemiological importance (Feliciangeli *et al.* 2007; Fitzpatrick *et al.* 2008). The frequent colonization of houses by sylvan populations of *R. prolixus* and their morphological confusion with *R. robustus*, make difficult its surveillance and control (Feliciangeli *et al.* 2007; Fitzpatrick *et al.* 2008). On the other hand, the sylvan species, *Rodnius robustus*–like*,* is morphologically indistinguishable to *R. robustus* and it might represent the same species previously reported in Ecuador (Márquez *et al.* 2011) by Abad-Franch and Monteiro (2005).

In domestic environments, the stability of food supply and protection from climatic extremes seem to have demographic consequences on reproduction, making populations achieve high densities (Schofield *et al.* 2006). The food supply (Gomez-Nuñez 1964; Aldana *et al.* 2001) and abiotic factors such as temperature and humidity (Silva and Silva 1988; Luz *et al.* 1999; Rocha *et al.* 2001) affect insect development. In both the species studied here, all five instars including adults are obligatory haematophagous insects and they are unable to moult without feeding. Nymphs need to feed at least once *ad repletionem* to attain the following stage (Luz *et al.* 1999). Hence, the low feeding frequency may prolong the duration of life cycle of these species, which ranges from 73 to 206 days in *R. prolixus* (Arévalo *et al.* 2007) and from 127 to 224 days in *R. robustus* (Tonn *et al.* 1976) for insects fed on hen blood. On the other hand, the high-population-density conditions seem produce competence for food amongst insects and a consequent lower consumption of blood per individual (Dujardin *et al.* 1999).

Therefore, both population density and feeding frequency have been used to simulate sylvan and domestic conditions in the laboratory, assuming that the low levels of these factors occur in sylvan habitat, whereas the highest levels are commonly observed in domestic conditions (Caro-Riaño *et al.* 2009; Márquez 2011). These two factors induce metric changes in *Rhodnius pallescens* (Caro-Riaño *et al.* 2009) and *R. robustus*–like (Márquez 2011) and they seem to explain the size modifications observed from the transition from sylvatic to domestic habitat (Zeledón *et al.* 1970; Zeledón 1981; Dujardin *et al.* 1997; 1999; Jaramillo *et al.* 2002; Feliciangeli *et al.* 2007; Rodríguez *et al.* 2007). Additionally, these two factors also induce changes in wing shape in *R. prolixus* and *R. robustus*–like (Márquez 2011).

The comparison of FA values between domestic and domiciliary species of *Triatoma sordida*, *Triatoma infestans* (Dujardin *et al.* 1999) and *Rhodnius pallescens* (Jaramillo 2000) has led to the conclusion that FA in wing size is higher in domiciliary environments than in the sylvan ones, because of a lower selection pressure on individuals with reduced flying activity in domiciliary conditions (Dujardin *et al.* 1999; Schofield *et al.* 2006). On the other hand, other studies

made with *Triatoma dimidiata* have shown that FA can be similar amongst domestic and sylvan populations (Menes-Hernández 2004) and FA can even change amongst domestic populations (Lehmann *et al.* 2005) and amongst non-domiciliated populations seasonally infecting houses (Nouvellet *et al.* 2011). However, it is not clear whether the individuals submitted to change in habitat may be subject to synergistic stresses of environmental (population density, feeding frequency, food source, etc.) and genetic origin (genetic drift). Besides, the role of the FA on the metric changes described above is unknown.

In this context, this work measured the levels of asymmetry in wing size and shape in two species of *Rhodnius* to test in the laboratory whether changes in feeding frequency and population density may represent stressful conditions that can cause developmental instability in both species. Thus, our prediction is that developmental instability (more asymmetric individuals) may be higher when the species are forced to grow under high population densities, different feeding frequencies or the combined effect of both treatments, as a signal of environmental stress (Polak 2003; Soto *et al.* 2008).

2. Materials and methods

2.1 *Insects*

Two species of Triatomine were used in this study, a domestic habitat species, *Rodnius prolixus* (Stål 1859), and a sylvatic habitat species, *Rodnius robustus*–like (Márquez *et al.* 2011). Both species had been kept in the laboratory for more than 10 years, growing under controlled conditions of lighting, temperature (25±2°C), relative humidity (80±5%) and bimonthly feeding on hens. *R. prolixus* colony was established by the laboratory group of Biología y Control de Enfermedades Infecciosas in 1991 from specimens captured in human dwelling at the municipality of Coyaima, Department of Tolima, Colombia. In addition, *R. robustus*–like colony was established in 1999, from a female collected in palm trees at the municipality of Puerto Asís, Putumayo, Colombia. Morphologic characterization of both species was performed according to the criteria described by Lent and Wygodzinsky (1979). Furthermore, the genetic identification of both species of insects was verified with a molecular characterization based on sequencing analysis of the mitochondrial gene *Cytochrome b* and the D2 variable region of the 28S RNA (Márquez *et al.* 2011).

2.2 *Experimental design*

Three isofamilies per species were established under laboratory conditions. Each family was produced by crossing one male with five females. For these crosses only virgin adults

were obtained by using V-instar sexed nymphs of each species. A total of 1200 half-sib first instar nymphs were used in this work: 720 of *R. prolixus* individuals and 480 individuals of *R. robustus*–like. All nymphs were fed on hen blood and were randomly distributed in glass jars (230 mL, 7.1 cm diameter × 7.6 cm height) to carry out four treatments: the first treatment consisted of 40 nymphs/jar that were fed weekly (W40), the second treatment was composed by another group of 40 nymphs/jar that were fed monthly (M40), the third treatment consisted of 80 nymphs/jar fed weekly (W80) and finally the fourth treatment consisted of 80 nymphs/jar fed monthly (M80). The treatments M40 (low population density and low feeding frequency) and W80 (high population density and high feeding frequency) can be envisaged as an analog between sylvatic and domestic environments. Given that the number of first instar nymphs obtained from the three isofamilies was low in the *R. robustus*–like line, the replicates used in our experiments differed between the species. We obtained three replicates for the *R. prolixus* line and two replicates for the in *R. robustus*–like line. Both species lines were maintained in an insect room under controlled conditions with a photoperiod of 12:12, a temperature of 25±2°C and relative humidity of 80±5%. The feeding period on the hens were about 105 min per individual, with a feeding frequency according to the treatments. Hens were kept according to the Research Ethic Committee based on the resolution number 008430 of the Ministry of Health of Colombia. Survival was measured as the number of adult insects per treatment. Observations were made until the last nymph V-instar per each treatment reached adult stage or died. The developmental period from first instar until adult for insects fed weekly ranged from 2.5 to 6.9 months in *R. prolixus* and from 3.8 to 6.8 months in *R. robustus*–like. In insects fed monthly, this period ranged from 5 to 9.4 months in *R. prolixus* and from 5 to 8 months in *R. robustus*–like. The effects of population density and feeding frequency on survival were investigated by a two-way ANOVA, including population density and feeding frequency as the main effects and survival as dependent variable.

2.3 *Geometric morphometrics*

Because of high mortality rate of these insects, a total number of 1122 right and left wings were dissected, from which 772 belonged to the *R. prolixus* line and 350 belonged to *R. robustus*–like line. All wings were mounted by using standard techniques and photographed with a Nikon 990 digital camera that was adapted to a stereomicroscope Nikon SMS 800. Nine landmarks of type I (Bookstein 1991) were identified on the wing hemelytron membrane part (figure 1). To detect potential peripheral optic distortions, we compared two sets of photographs taken on the same set of wings at different positions. Digital precision
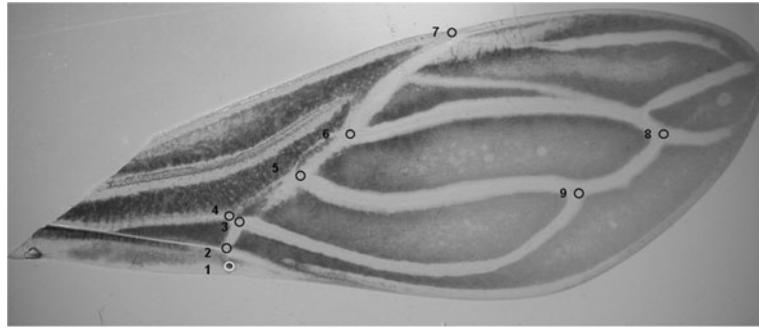
Figure 1. Landmarks type I measured as coordinates of wings of *R. prolixus* and *R. robustus*–like. Numbering on the landmarks denotes the arrangement followed to obtain the coordinates. Landmark 1 corresponds to the '*coaptor*' located on the '*clavum.*' Landmarks 2–7 are located along the '*corium*-membrane junction' of the wing, and landmarks 8 and 9 are on the membranous part.

was estimated with 'Repeatability' index (R=individual variation/total variation) by using a model II one-way ANOVA on repeated measures (Arnqvist and Märtensson 1998).

Isometric estimator known as centroid size derived from the coordinates was used as the size variable of the wing. Centroid size is defined as the square root of the sum of the squared distances between the centre of the configuration of landmarks and each individual landmark (Bookstein 1991). Raw coordinates were submitted to superimposition algorithm of Generalized Procrustes Analysis to produce '*partial warps*' scores (Rohlf 1990; Rohlf and Slice 1990). Both non-uniform and uniform components were used as shape variables of the wing.

2.4 *Asymmetry assessment*

To analyse the bilateral asymmetry in size, phenotypic variation was divided in three components, the intra-individual variation, the inter-individual variation and the error components by using a two-way ANOVA design (Leamy 1984; Palmer and Strobeck 1986; Palmer 1994). In this analysis, the 'individual' component is a random factor that establishes individual variations in size or shape; 'sides' is a fixed factor that was considered the estimator of the directional asymmetry (DA); the interaction between the individual × side components provides a measure of the non-directional asymmetry and the residual variance component gives an estimation of the measurement of the error. To detect departures from normality in the samples and the presence of antisymmetry, Shapiro–Wilks, Kurtosis and Skewness statistics were estimated for the signed differences in size between sides in each treatment combination.

FA differences in size among samples were contrasted using the absolute value of difference between right and left side (R−L), following Palmer and Strobeck (1992) procedure. As directional asymmetry was significant (see section 3.1), the method recommended by Palmer (1994) was used to correct

the FA estimation. Briefly, each individual sample was corrected for each side size by subtracting half of the mean of R−L [mean (R−L)/2] from the large side and adding it to the smaller side (Palmer 1994). Comparisons among treatments were made by using Kruskal–Wallis test and pairwise comparisons were made by using Mann–Whitney test. The corrections for the significant P values were performed with a Bonferroni sequential method (Sokal and Rohlf 1995). To assess for sample FA size dependence, a simple lineal regression was carried out taking into account logarithm of the variance of the side difference [log(var(R−L)] with respect to each individual average wing size mean (Palmer 1994).

The possible transitions from FA to DA, which were proposed as development instability indicators (Graham *et al.* 1993a; Graham *et al.* 1993b; Soto *et al.* 2008), were explored with Mantel test (Mantel 1967) and statistical significance was determined with 10,000 permutations.

To examine asymmetry in shape, the procedure described by Klingenberg and McIntyre (1998) was used in this study. To assess the possibility of antisymmetry, scatterplots of left–right coordinate differences for each landmark after Procrustes superposition were visually inspected for clustering of the values (Klingenberg and McIntyre 1998; Debat *et al.* 2000). FA differences on each treatment were compared by using a multi-factor MANOVA test. FA differences within and among treatments in each genotype were tested according to Soto *et al.* (2008). In this case, to prevent the DA effect (explained by 'side' factor), squared mean of the individual × side interaction and its corresponding degrees of freedom were obtained from multi-factor MANOVA test.

2.5 *Software*

ANOVA tests used here to analyse size and shape asymmetries were performed on ASI program incorporated in CLIC package V. 70 (Dujardin 2013). The other statistical tests and assumptions were carried out on Statgraphics plus program V. 2.1.

Finally, Mantel program for Windows V. 1.19 (Cavalcanti 1988) was used to perform a Mantel test (Mantel 1967).

3. Results

3.1 Repeatability

Congruence between the two sets of measurements obtained from the same set of photographs of the wings showed fairly good estimations for centroid size (R= 0.99), as well as for the four relative warps (RW1, RW2, RW3 and RW4, with R= 0.98, 0.95, 0.92 and 0.91, respectively) representing 83% of the shape variation. Measurement error increased on the following relative warps values as observed by Arnqvist and Märtensson (1998) and Caro-Riaño *et al.* (2009) in other insect species.

3.2 Survival

Decrease of survival at high population density was highly significant: In *R. prolixus* survival decreased from 82.5% to

46.67% ($F_{(1, 10)}$=9.30, *P*=0.019) and in *R. robustus*–like from 63.75% to 25.83% ($F_{(1, 11)}$= 9.17, *P*=0.016). In contrast, neither feeding frequency (*R. prolixus*: $F_{(1, 10)}$=0.32, *P*=0.588; *R. robustus*–like: $F_{(1, 11)}$=0.04, *P*=0.847) nor the interaction population density × feeding frequency effects significantly affected survival in both species (*R. prolixus*: $F_{(1, 10)}$=0.32, *P*=0.588; *R. robustus*–like: $F_{(1, 11)}$=1.43, *P*=0.265).

3.3 Wing size asymmetry

Descriptive statistics of wing size asymmetry are summarized in the table 1. The signed differences in R−L sizes were normally distributed in both species, in all treatments (table 1).

The results obtained in the ANOVA analysis showed that both DA and FA of the wing size were highly significant in all treatments in *R. prolixus* (*P*<0.0001; table 2). Highly significant levels of DA and FA were also found in *R. robustus*–like (*P*<0.0001) except in three treatments where DA was no significant (females: W80; males: W40

Table 1. Descriptive statistics of wing size asymmetry

Treatment	N	Mean CS		R−L			Shapiro–Wilks Test		|R−L|
		R	L	Mean	Skewness	Kurtosis	W	*P*	Mean
R. prolixus									
Females									
M40	41	8.589	8.598	−0.008	−0.220	0.879	0.988	0.960	0.060
M80	43	8.438	8.433	0.005	1.379	1.274	0.974	0.580	0.080
W40	55	8.674	8.671	0.002	1.514	0.012	0.965	0.204	0.068
W80	58	8.579	8.598	−0.015	−0.572	−0.285	0.983	0.810	0.068
Males									
M40	23	7.726	7.750	−0.019	1.097	0.515	0.968	0.600	0.094
M80	50	7.651	7.686	−0.004	0.096	0.147	0.980	0.599	0.118
W40	43	8.086	8.097	−0.009	0.364	0.422	0.993	0.998	0.082
W80	64	7.856	7.854	0.005	−0.541	1.451	0.985	0.850	0.068
R. robustus–like									
Females									
M40	13	9.570	9.686	−0.072	0.279	−0.471	0.971	0.862	0.164
M80	21	9.666	9.549	0.044	−0.041	−0.347	0.972	0.758	0.142
W40	20	9.403	9.436	−0.007	0.240	−0.356	0.987	0.980	0.086
W80	16	9.104	9.111	−0.013	−0.882	1.655	0.955	0.556	0.079
Males									
M40	23	8.720	8.702	0.001	−0.189	0.649	0.954	0.353	0.097
M80	30	8.519	8.632	−0.008	−0.223	−0.032	0.977	0.782	0.055
W40	25	8.536	8.541	−0.003	−0.702	2.710	0.932	0.102	0.061
W80	24	8.377	8.386	−0.008	0.107	−0.996	0.958	0.402	0.032

M40: density 40 monthly feeding; M80: density 80 monthly feeding; W40: density 40 weekly feeding; W80: density 80 weekly feeding.

Table 2. Summary of ANOVA on wing size of *R. prolixus* and *R. robustus*–like

Effect	R. prolixus						R. robustus–like					
	Females			Males			Females			Males		
	MS	df	F	MS	df	F	MS	df	F	MS	df	F
M40												
Side	20.4	1	138.8*	14.1	1	64.9*	1.3	1	10.8*	0.2	1	1.3n
Side×Individual	1.7	40	11.82*	2.1	24	9.8*	1.8	12	14.5*	1.5	22	8.4*
Error	0.1	164		0.2	100		0.1	52		0.2	92	
M80												
Side	33.6	1	202.6*	29.2	1	228.6*	1.8	1	12.9*	17.7	1	183.4*
Side×Individual	1.5	42	9.01*	2.9	54	23.1*	1.4	20	10.2*	2.0	30	20.6*
Error	0.2	172		0.1	220		0.1	84		0.1	124	
W40												
Side	55.6	1	251.4*	17.1	1	104.6*	40.9	1	236.1*	0.3	1	1.8n
Side×Individual	3.0	54	13.4*	1.7	42	10.4*	6.8	20	39.2*	1.9	25	10.8*
Error	0.2	220		0.2	172		0.2	84		0.2	104	
W80												
Side	28.4	1	155.2*	9.7	1	49.6*	0.1	1	3.3n	15.7	1	75.7*
Side×Individual	2.4	58	13.09*	2.5	64	13.0*	1.2	16	69.7*	4.2	23	20.4*
Error	0.2	236		0.2	260			68		0.2	96	

W40, M40: Weekly and monthly feeding at density 40. W80, M80: Weekly and monthly feeding at density 80. MS: Mean squares multiplied by 10e5, df: degree of freedom, n: non-significant difference. *$P < 0.00001$.

and M40; table 2). Error measure was 18.6 times smaller on average than the levels of FA.

Sexual differences in the levels of FA varied across treatments: they were not significant at low population density, whereas at high population densities, females showed higher levels of FA than males (figure 2). Due to these sexual differences, individuals of both sexes were analysed separately to compare differences amongst treatments.

In the high-population-density treatment, we found a significant increment in the levels of FA for the wing size in *R. robustus*–like females reared on high feeding frequency (W40–W80: $P=0.004$; figure 3). In contrast, the high-population-density treatment did not affect the levels of FA in *R. robustus*–like females reared on low feeding frequency, in *R. robustus*–like males ($P=0.146$) and in either sex of *R. prolixus* ($P≥0.122$) (figure 3). Changes in feeding frequency or the combination of high values of population density and feeding frequency did not affect the levels of FA for either sex of both species. The levels of FA were not dependent on size due to the lack of a relationship between them ($P>0.10$). Right and left wings showed isometric growth (regression of right versus left wing size showed a slope roughly 1; $P<0.0001$ in all contrasts). In addition, no significant correlation between FA and DA was found for either *R. prolixus* ($r=0.215$, $t=0.522$ and $P=0.699$) or *R. robustus*–like ($r=−0.058$, $t=−0.709$, $P=0.239$).

3.4 *Wing shape asymmetry*

Distribution of |R−L| difference after Procrustes superposition did not show any tendency to clustering. ANOVA Procrustes analysis, used to contrast asymmetry in shape, revealed a significant effect of both DA and FA in all treatments ($P<0.0001$ in all cases).

FA degree in shape between sexes was invariable in *R. prolixus*, while in *R. robustus*–like it changed across treatments (figure 4). Therefore, also in this case, sexes were analysed separately to contrast differences amongst treatments. FA degree in wing shape in both species did not change with treatments, except in a single comparison made in males (table 3).

4. Discussion

The FA of Triatominae wings have shown in previous studies their tendency to increase in individuals from domestic environments (Dujardin *et al.* 1999). However, in these studies it was not clear whether the differences in the levels of FA between sylvatic and domestic habitats were attributable to the effect on environmental differences between treatments (i.e. population density, feeding frequency, food source, etc.) or to the effect of genetic stress (i.e. genetic
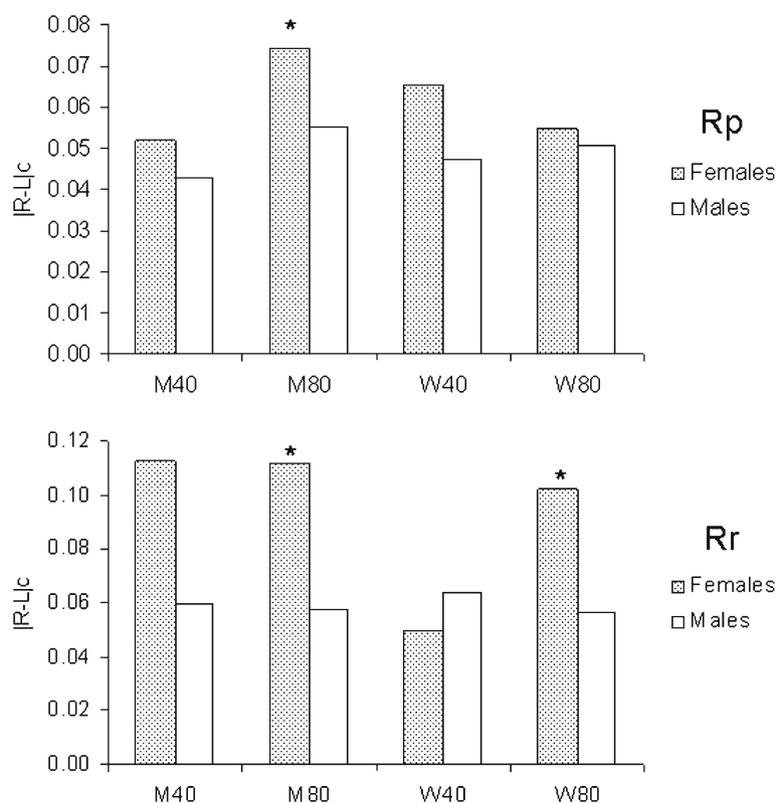
Figure 2. Sexual dimorphism of fluctuating asymmetry degree in wing size of *R. prolixus* (Rp) and *R. robustus*–like (Rr). R−L c: Mean unsigned differences in size between right and left wings corrected by directional asymmetry; M40: density 40 monthly feeding; M80: density 80 monthly feeding; W40: density 40 weekly feeding; W80: density 80 weekly feeding. * denotes statistical significance between sexes ($P = 0.002$).

drift, inbreeding) of the species involved in those works. The use of half-sib design in this work allowed us to remove differences in the degree of genetic drift amongst treatments and to focus on two environmental effects: population density and feeding frequency.

DA and FA of wing size and shape of the *Rhodnius* wings were evident in all assessed treatments. DA has been interpreted as an evidence of development instability when transition from FA to DA is observed (Graham *et al.* 1993a, b; Soto *et al.* 2008). Nevertheless, the results of this work did not provide enough evidence for this hypothesis since FA was not correlated to DA and nor was it consistent with feeding frequency and population density changes. DA seems to be a common trait found in insects that are reared under laboratory conditions; examples of them are the established laboratory strains of *R. pallescens* (Jaramillo 2000) and *Drosophila* (Klingenberg *et al.* 1998; Hoffmann *et al.* 2005). An alternative explanation is that individuals that did not exhibit such asymmetry were not capable of surviving under the assessed conditions.

As that DA can produce overestimations of FA and there-fore on development instability (Graham *et al.* 1998),

Palmer's (1994) procedure was used in this work to correct FA estimation (section 2.4). In all cases, data error values were lower than FA, suggesting that this error did not pro-duce a bias on the measurement of the FA obtained here. In addition, FA comparisons amongst treatments were performed separately for each sex as the magnitude of FA sexual dimorphism changed under different population den-sity and feeding frequency conditions. The level of FA was significantly greater in females compared to males, which is concordant with the results found in *T. infestans* (Dujardin *et al.* 1997). However, this outcome contrasts with the results found in *T. dimidiata*, where males showed greater levels of FA compared to females (Lehmann *et al.* 2005; Nouvellet *et al.* 2011).

The FA sexual dimorphism in *T. dimidiata* has been proposed that result from differential use of flight between sexes (Nouvellet *et al.* 2011) based on two observations: (1) similar sexual differences has been reported in other insects and in birds, which showed that increased wing size and lower FA provided better flight abilities in these taxa and (2) females initiate flight more frequently than males (Lehane and Schofield 1982; Williams and Schofield 1985; Gurevitz
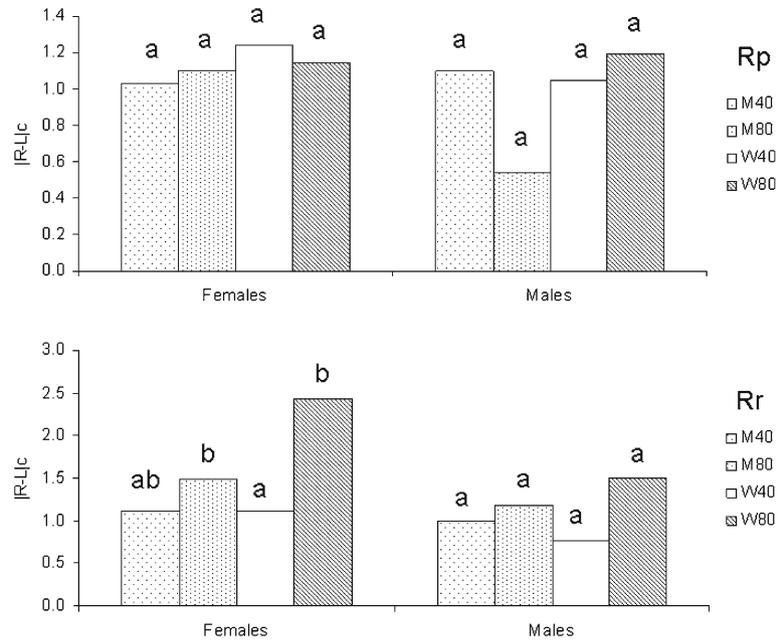
Figure 3. Mean unsigned differences in size between right and left wings in females and males of *R. prolixus* (Rp) and *R. robustus*–like (Rr). Mean unsigned differences were standardized and corrected by directional asymmetry (R−L c). M40: density 40 monthly feeding; M80: density 80 monthly feeding; W40: density 40 weekly feeding; W80: density 80 weekly feeding. Letters denote homogeneous group according to the Mann–Whitney test. *P*-values were corrected with a Bonferroni sequential method.

et al. 2006). The differential use of flight has been also found in *R. prolixus*, where males can selectively perform upwind flight induced by a pheromone released by females metasternal glands (Pontes *et al.* 2008). However, the observations made in these two studies do not seem to explain our results since sexual differences in FA of *Rhodnius* changed under population density and feeding frequency conditions, suggesting a plastic response of this trait to the treatments. Furthermore, other studies based on laboratory-reared bugs did not find significant sex differences in flight of *T. infestans* (Lehane *et al.* 1992) and *R. prolixus* (Minoli

and Lazzari 2006), suggesting that other factors contribute to differences in flight between the sexes.

The nutritional stress induced by the high population density seems affect the levels of FA in wing size of females and wing shape of males of *R. robustus*–like fed weekly and the wing shape of males of *R. prolixus* fed monthly (comparison M80–W40; table 3). Increased levels of FA under high-density conditions were also found on several bilateral traits including wing length in *D. melanogaster* (Imasheva *et al.* 1999) and *D. ananassae* (Vishalakshi and Singh 2009). Additionally, sexual differences in levels of FA in response
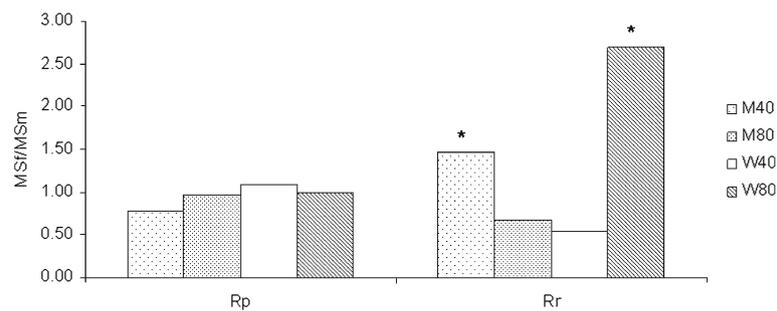
Figure 4. Sexual dimorphism of fluctuating asymmetry degree in wing shape of *R. prolixus* (Rp) and *R. robustus*–like (Rr). M40: density 40 monthly feeding; M80: density 80 monthly feeding; W40: density 40 weekly feeding; W80: density 80 weekly feeding. * denotes significant differences ($P \leq 0.002$).

Table 3. Pair-wise mean square (MS) comparisons of treatments in *R. prolixus* and *R. robustus*–like male and female

Contrasts		*R. prolixus*				*R. robustus*–like			
		MS	df1	df2	*P*	MS	df1	df2	*P*
Females									
Density (D)	M40-M80	0.750	560	588	1.000	1.087	168	280	0.269
	W40-W80	0.897	756	812	0.936	0.289	280	224	1.000
Feeding (F)	M40-W40	0.808	560	756	0.996	0.893	168	280	0.789
	M80-W80	0.966	588	812	0.673	0.237	280	224	1.000
D × F	M80-W40	1.077	588	756	0.169	0.821	280	280	0.950
	M40-W80	0.724	560	812	1.000	0.258	168	224	1.000
Males									
Density (D)	M40-M80	0.931	336	756	0.775	0.500	308	420	1.000
	W40-W80	0.828	588	896	0.994	1.472	350	322	0.000*
Feeding (F)	M40-W40	1.125	336	588	0.109	0.321	308	350	1.000
	M80-W80	1.000	756	896	0.499	0.944	420	322	0.710
D × F	M80-W40	1.208	756	588	0.008*	0.642	420	350	1.000
	M40-W80	0.931	336	896	0.780	0.472	308	322	1.000

M40: density 40 monthly feeding; M80: density 80 monthly feeding; W40: density 40 weekly feeding; W80: density 80 weekly feeding; df: degrees of freedom; *P*: statistical significance. * denotes significant differences.

to nutritional stress were also found in stalk-eye flies, *Cyrtodipsis dalmanni* (Bjorksten *et al.* 2000), where males exhibited differences in FA depending to the rearing media, whereas females did not show such an effect.

However, contrary to our expectations, in most cases our study demonstrated unaffected levels of FA by nutritional stress provided by high population density. Similarly, the individuals submitted to the treatments analogous to sylvan and domestic conditions (M40 and W80) also showed unaffected levels of FA. This outcome cannot be regarded a consequence of a modest effect of treatments because the nutritional stress provided by high population density decreased significantly the survival for either sex of both species. On the other hand, all insects that reached the adult stage were included in our analysis, making unlikely an underestimation of asymmetric individuals by a prolonged development of insects. Furthermore, in this study the low feeding frequency, instead the high population density, prolonged the developmental period of insects. Such behaviour results from the inability of these insects to moult without feeding and the needing to feed at least once *ad repletionem* to attain the following stage (Luz *et al.* 1999). Although the mortality of asymmetric individuals before reaching the adult stage can not be disregarded as a plausible explanation, this outcome indicate that the nutritional stress provided by high population density in *Rhodnius* may have negligible effects on FA.

The unaffected levels of FA in response to nutritional stress are concordant with results found in other taxa (Gronkjaer and Sand 2003; Searcy *et al.* 2004; Stige *et al.*

2004) including insects (Bjorksten *et al.* 2000; Woods *et al.* 2002; Vijendravarma *et al.* 2011). Furthermore, the outcome between the treatments analogous to sylvan and domestic conditions is concordant with that found in *T. dimidiata* (Menes-Hernández 2004), although in that study the insects were not reared under controlled conditions. However, our results contrast with those found in *T. sordida* whose domestic populations showed greater levels of FA than the sylvan ones (Dujardin *et al.* 1999). The discrepancy between this study and Dujardin *et al.* (1999) may result from the differences in species studied, the rearing conditions of the insects (laboratory vs field conditions) and possibly the degree of genetic drift between individuals compared. This explanation is supported by the idea that the variation of FA may be specific to the trait measure, organism and populations studied (Vijendravarma *et al.* 2011), thus limiting the ability to generalize patterns amongst different studies.

In conclusion, our results reflect both inter-specific and sexual dimorphic differences in FA of wing size and shape induced by nutritional stress, suggesting different abilities of the genotypes and sexes of two sylvatic and domestic genotypes of *Rhodnius* to buffer these stress conditions. Additionally, the analysis of data present herein and those found by Márquez (2011) indicate that the phenotypic variations induced by changes in population density and feeding frequency in wing size and shape in both species (Márquez 2011) are not explained by developmental noise. Therefore, our results confirm that the variation in wing size and shape in response to treatments constitute a plastic response of these genotypes to population

density and feeding frequency. A similar result was found for several wing measurements of female *D. melanogaster*, which showed unaffected levels of FA and plasticity in response to nutritional stress (Vijendravarma *et al.* 2011). The effect of these factors on changes of the wing size had been previous reported in *R. pallescens* reared under controlled conditions (Caro-Riaño *et al.* 2009), but its relationship with FA had not been previously explored. The basic biological information provided for these species serves as reference for future studies in evolution and the relationship with the epidemiological surveillance of Chagas disease in the distribution area of these vectors.

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