Wing shape divergence between *Rhodnius prolixus* from Cojedes (Venezuela) and *Rhodnius robustus* from Mérida (Venezuela)

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Abstract

The existence of *Rhodnius robustus* as a species distinct from *Rhodnius prolixus* has long been the main epidemiological question about Chagas disease transmission in Venezuela and surrounding countries. These two taxa are morphologically and genetically very similar, but only *R. prolixus* is assumed to colonize houses and transmit Chagas disease to humans. *R. robustus* is assumed to be an exclusively sylvatic species, restricted to palm trees. If *robustus* and *prolixus* are actually the same species, the theoretical possibility exists of sylvatic specimens invading houses, even after insecticide application, and a control strategy similar to that of the successful Southern Cone Initiative against *Triatoma infestans* would be difficult to consider. Since no valid alternative control strategy exists, the answer to this biological question could be decisive about the future of vector control in this region. Although we believe genetic techniques are best suited to define species boundaries, we present here an example of the relevance of modern morphometrics in dealing with such an issue. Using both traditional and geometric morphometrics, we compared the wing size and shape in both sexes of these two taxa reared in the same laboratory for one generation. *R. robustus* specimens were collected from palm trees in the state of Mérida (Venezuela), and *R. prolixus* were collected from houses in the state of Cojedes (Venezuela). Our study provided no argument to question their specific status. Even after one generation of living in the same laboratory conditions, the two lines showed clear size differences, divergent allometric trends, and significant allometry-free differences in shape. These results suggest that *R. robustus* (Mérida, Venezuela) and *R. prolixus* (Cojedes, Venezuela) are distinct evolutionary units. Due to the epidemiological importance of this question, further studies in other geographic areas of Venezuela are required to accurately define the relationships of *R. robustus* and *R. prolixus*.
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1. Introduction

Rhodnius prolixus Stal (Hemiptera, Reduviidae, Triatominae) is one of the main Chagas disease vectors in Latin America. It is recognized as a domestic and peridomestic species, and most laboratory isolates derive from collections from houses and/or chicken coops. However, sylvatic populations mainly in palm tree crowns–have been reported in parts of Venezuela since the work of Gamboa (1962). Palm tree crowns in these regions are also considered the main habitat of morphologically similar *Rhodnius robustus* Larousse, with the two species distinguished only by the lighter colored tibia of the older nymphs and minor differ-

ences in the basal plate struts of the male genitalia (Lent and Wygodzinsky, 1979).

Isoenzyme studies found no consistent differences between these two species, and no reproductive isolation was apparent in laboratory crosses (Harry et al., 1992a,b; Harry, 1993; Barrett, 1996; Solano et al., 1996), so that the specific status of *robustus* as a distinct taxon has been questioned (Harry, 1993; Barrett, 1996). However, recent mtDNA sequence data do indicate differences between individuals from houses (defined as *prolixus*) and individuals from palm-trees (defined as *robustus*) (Lyman et al., 1999; Monteiro et al., 2000), and the domestic populations show different salivary protein profiles compared to morphologically similar populations from palm trees in northern Brazil (Soares et al., 1998).

The status of these two entities is of epidemiological importance because the domestic populations of *R. prolixus* are

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a primary target for Chagas disease vector control interventions, whereas the palm tree populations–with little contact with humans–may have little epidemiological relevance (but see Feliciangeli et al., 2002). However, if there is significant gene flow between the two, then interventions against the domestic vectors might be severely compromised by reinvasion from the sylvatic foci.

It has been suggested that *prolixus* may represent the domestic derivative of *robustus* (Schofield and Dujardin, 1997), subsequently dispersed in association with human population movements. Recalling the arguments of Falconer (1981) that the first changes concerned in micro-evolution are changes of metric characters, we reasoned that, even if recent, the separation between *prolixus* and *robustus* would have already induced metric differences such that the two forms could not be considered as merely allometric extensions of each other. We therefore examined this idea using both traditional and geometric morphometrics, both as a contribution to resolving the taxonomic question and also as an introduction to the application of geometric morphometrics to the study of Triatominae.

We examined wing geometry in relation to six landmarks using the thin-plate spline method (Bookstein, 1990), which allows visualization of shape variation and allometric trends within and between taxa. Instead of measurements of distances between landmarks, the geometric method uses coordinates of landmarks. This has several advantages (Rohlf and Marcus, 1993), providing a precise quantification of shape differences and allowing their visualization through the classical deformation grids suggested by D'Arcy-Thompson. By constructing separate variables for general size and shape variation, it also allows allometry to be visualized and tested (Bookstein, 1991; Krzanowski, 1988; Rohlf, 1996; Baylac and Penin, 1998). If necessary, overall shape changes may also be decomposed into a global "uniform" and multiple local "non-uniform" shape changes. Using the D'Arcy-Thompson grid metaphor, non-uniform shape changes are local deformations distorting parts of the grid, while uniform changes are the set of transformations which graphically preserve the parallelism of the grid lines. Although purely mathematical, such decomposition may point out some particular shape changes, and has proven to be useful at different taxonomic levels (Baylac and Penin, 1998; Swiderski, 1993; Baylac and Daufresne, 1996).

For the hypothesis that one group is a larger or smaller representative of the other, it was expected that allometric changes alone were responsible for possible differences in shape. This would justify comparison of the allometric trends and examination of the effect of removing them on the metric differences observed. Allometries were compared using thin-plate splines method, their removing used traditional techniques as good models have been developed for this purpose based on distances measurements–for a review, see Klingenberg (1996). To remove the allometric influence on shape changes, i.e. to evidence possible shape differences unexplained by size variation (growth), we applied

the technique combining the orthogonal projection method (Burnaby, 1966) and the common principal analysis (Flury, 1984; Airoldi and Flury, 1988; Klingenberg, 1996).

2. Material and methods

2.1. Insects

R. prolixus was collected intradomiciliary at the Municipio San Carlos (NW J.A. Brabo) (La Sierra, State of Cojedes, Venezuela)–forty kilometers north of San Carlos city. *R. robustus* was collected from palm trees (*Acrocomia aculeata* and *Scheelea macrocarpa*) at the Municipio Zea (locality Caño Tigre, State of Mérida, Venezuela). Both samples were reared in the same insectarium for one generation after capture, and then submitted to metric analysis. In total, 102 wings were examined: 38 of *R. prolixus* (18 female and 20 male wings) and 64 wings of *R. robustus* (48 female and 16 male wings) (Table 1).

2.2. Metric data (Fig. 1)

Camera lucida drawings of the wings were made on a microscope at a magnification that allowed maintenance of

Table 1
Material examined

	R. prolixus	*R. robustus*
Origin	La Sierra (Cojedes) 9°53′56″N, 68°35′14″W	Caño El Tigre (Mérida) 8°26′30″N, 71°46′18″W
Habitat	Houses	Palm trees
MAT[a]	23 °C	24 °C
MRH[b]	0.70	0.75
ALT[c]	1200 m	250 m
N	38 (18F[d] and 20M[e])	64 (48F and 16M)

[a] MAT: mean annual temperature.
[b] MRH: mean (annual) relative humidity.
[c] ALT: altitude.
[d] F: female wings.
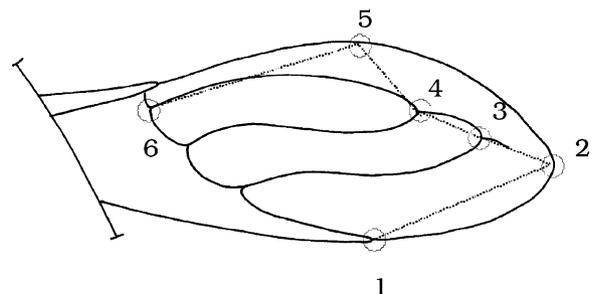[e] M: male wings.

Fig. 1. Dorsal view of the left wing of *Rhodnius* sp. Circles and associated numbers indicate the landmarks used, dashed lines between them the distance measurements taken. For instance, W15 means the distance between landmarks W1 and W5. These landmarks are located on the anterior (W1)–posterior (W5) axis of the wing.

a consistent plane of focus to control distortion. On the membranous part of the hemelytra, we identified six landmarks: four of "type I", denoted W1, W3, W4 and W6 (tissue intersections), and two of "type II", denoted W2 and W5 (Bookstein, 1990). The coordinates of landmarks were digitized using TPSdig (version 1.15; Rohlf, 1999).

We also measured seven, non-redundant features of the fore wing (hemelytra) denoted: W12, W23, W34, W45, W56 (all distances describing the outline of the wing), W26 (an estimation of wing length), and W15 (an estimation of wing width).

2.3. Size variation

For comparing overall wing size between both groups, we used the isometric estimator known as "centroid size" derived from coordinates data. It is defined as the square root of the sum of the squared distances between the center of the configuration of landmarks and each individual landmark. Since centroid size is an isometric size parameter, the resulting superimposed shape coordinates did not necessarily remove any allometric change.

2.4. Shape variation

Shape variables were obtained through a geometric approach, as deformations ("partial warps") from a consensus configuration using the TPSrelw software (Rohlf, 1993, 1998b).

The raw coordinates are first superimposed using a Generalized Procrustes analysis (GPA) superimposition algorithm, whereby the sum of squared distances between each object and a reference configuration are iteratively minimized by translations and rigid rotations (Rohlf, 1990; Goodall, 1991, 1995). At each iteration the reference, which is taken as the mean configuration of the whole superimposed sample, is updated. Centroid size is eliminated from the superimposed coordinates by ratios. The uniform component of shape changes is expressed by two separate parameters: U1 and U2, which parameterize the dilatations and the shearing of the transformations (Bookstein, 1996). Together with the non-uniform component, ("partial warps", or "non-uniform parameters"), it recapitulates the overall shape changes which may be used in statistical analyses of shape. The thin-plate spline interpolation function may be used to apply the same changes to a squared grid providing a direct and quantitative implementation of the D'Arcy-Thompson transformation grids (Bookstein, 1991).

2.5. Allometries

The relationships between the thin-plate splines parameters and any variate (size, factorial axis, ecological variate etc.) may be analyzed by multivariate regression analysis (Rohlf and Marcus, 1993; Krzanowski, 1988; Mardia and Dryden, 1989). Regression parameters allow prediction and

visualization of the shape changes in relation to the specified variable. We used this approach to visualize the shape variation along the canonical axes, as well as to model the allometric patterns. These patterns were compared statistically by a multivariate analysis of covariance (MANCOVA), with total shape variables as dependent variables, taxa, centroid size and their interaction as independent variables.

2.6. Allometry-free differences

To remove a common allometric trend among a set of measurements, we used the common principal components model (Flury, 1984; Airoldi and Flury, 1988). In this model, a single component is taken to account for common allometric variation, provided the coefficients of the eigenvector are large and positive, and the correlations between this single component and each variable are positive and significant (Klingenberg and Zimmermann, 1992). In the space of the log-transformed measurements, conspecific individuals are expected to be found along the straight line defined by this single component. Metric variation orthogonal to this direction is allometry-free by construction, and describes allometry-free shape differences among individuals (Burnaby, 1966).

Using the 7 measurements described above, or all the combinations of 6 of them, the total sample (including both sexes, or considering the sexes separately) was not compatible with the hypothesis of a common allometric axis (i.e. with the model of a common principal component). We therefore explored the 21 possible combinations of 5 measurements, and identified only two sets compatible with a common allometric axis model, either in males or in females: the first one included three measurements describing the distal outline of the wing, its width and its length estimations (W12, W23, W34, W26, W15), the second one described the distal and proximal parts of the wing as well as its length (W12, W23, W45, W56, W26). The influence of within-group allometries was then removed by using residuals of an orthogonal projection of the data onto the first common principal component (Burnaby, 1966)–i.e., all the common principal components except the first one (Klingenberg, 1996). The resulting "allometry-free" variables were submitted to a canonical variate analysis (CVA), using the two putative taxa as groups irrespective of sex.

Since the interaction between sexes and group differentiation was significant (Table 2), sexes were also examined separately. In the same way as above, within-group allometries were removed and the resulting allometry-free, shape variables submitted to a discriminant analysis.

2.7. Software

Procrustes superimposition, thin-plate splines parameters, centroid sizes and graphical outputs were calculated and produced using the TPSRW and TPSREGR programs (Rohlf, 1998b,c). Statistical analyses were done using JMP® (SAS

Table 2
MANOVA

Var dep (Y)	W12, W23, W34, W26, W15	W12, W23, W45, W56, W26
Var indep (X)	sp., sex, sp.*sex	sp., sex, sp.*sex
Wilk's statistics:		
sp.	$P < 0.0001$	$P < 0.0001$
sex	$P < 0.0001$	$P < 0.0001$
sp.*sex	$P = 0.0075$	$P = 0.0132$

This multivariate analysis of variance (MANOVA) examines the possible interaction between sexes and group differentiation. Var dep (Y), dependent variable; Var indep (X), independent variables; sp, species; sp*sex, interaction between sex and species; W12, W23, W34, W45, W26, W56, W15, metric characters of the wing, for instance W12 is the distance measured between landmarks W1 and W2 (see Fig. 1).

Institute Inc., 1995), STATA® (Computing Resource Center, 1992) and NTSYS (Rohlf, 1998a). They included canonical variate analyses and MANOVA of the species and sex belongings, MANCOVA of the species and their centroid size. For traditional multivariate analyses, measurement data were log-transformed prior to analyses.

3. Results

3.1. Size variation

Non overlapping size differences between the wings of each sex completely separated *R. robustus*, with larger wings, from *R. prolixus*. There was a significant interaction between sexes and group differentiation (Fig. 2, Table 2).

3.2. Shape variation

Multiple regression of shape variables on isometric size revealed a significant ($P < 0.001$) allometric content that explained 36% of shape variation (detailed results not shown). This shape separation of the taxa seemed to be mainly of non-uniform kind, involving the distal and posterior parts of the wing (Fig. 3). In each sex, discriminant anal-
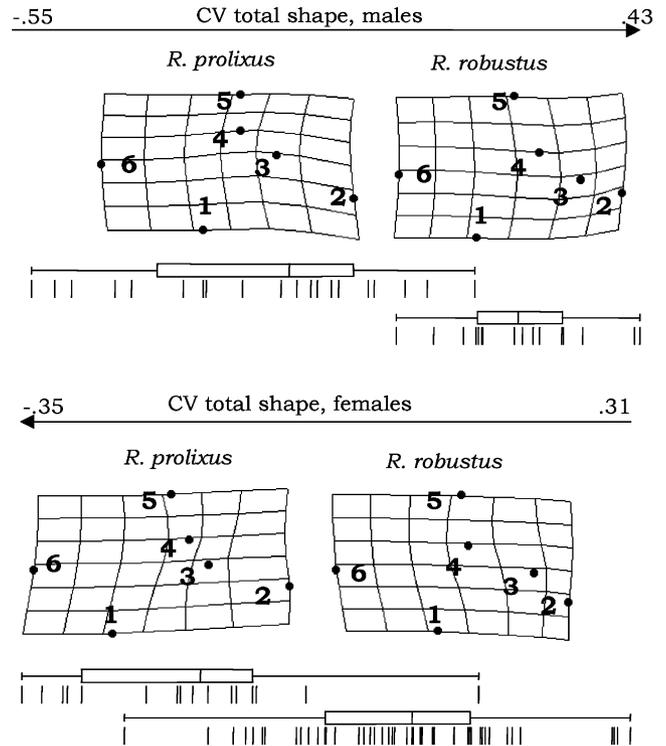

Fig. 3. Shape discrimination. Grid deformations corresponding to male (top) and females (bottom) *R. prolixus* and *R. robustus* along the discriminant factor derived from total shape, i.e. non-uniform and uniform parameters of shape changes. Males and females were analyzed separately, so that there was only one canonical factor (CV) by analysis: the dispersion of corresponding canonical values is shown by quantile plots under the grid deformations. In both sexes of *R. prolixus*, the apex of the wing (which is landmark W2) is more distant from the end of median and cubital veins (landmarks W3 and W4). These differences are not free of allometric influences (see text).

ysis gave satisfactory results for overall species comparison (Table 3).

3.3. Within-taxa allometric trends of the wing

The results of multivariate analysis of covariance (Table 4) on total geometric shape (with taxa and size as
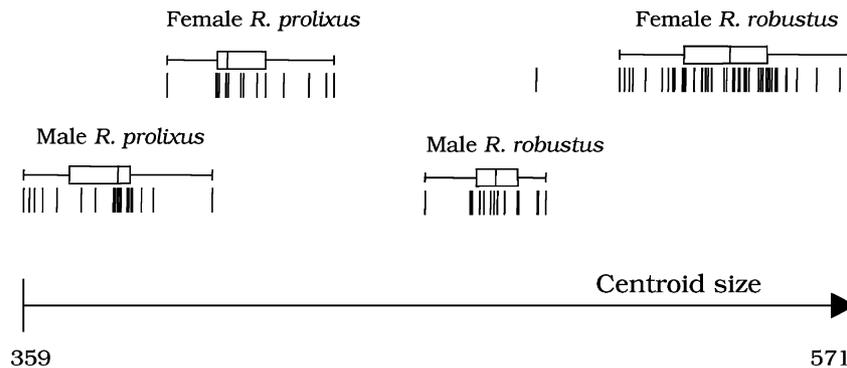

Fig. 2. Quantile plots showing in each sex the distribution of individuals along the isometric estimator of size (centroid size). Each box shows the group median separating the 25th and 75th quartiles, with the 10th and 90th quartiles shown as lines on the right and left sides of the box.

Table 3
Shape discrimination

Landmarks	W1, W2, W3, W4, W5, W6		
Shape variables	x1, y1, x2, y2, x3, y3, u1, u2		
	prolixus	*robustus*	Total, predicted
Females			
prolixus	15 (83%)	9	24
robustus	3	39 (81%)	42
2. Total, observed	18	48	66
Males			
prolixus	17 (85%)	0	17
robustus	3	16 (100%)	19
Total, observed	20	16	36

Re-classification of individuals according to discriminant analyses. W1, W2, W3, W4, W5, W6 = landmarks (see Fig. 1); x1, xy1, x2, y2, x3, y3, variables depicting the non-uniform component of shape; uniX and uniY, variables describing the uniform component of shape. In brackets, percent of correct attributions.

independent variables, and their interaction) indicated that both taxa did not react the same way to size variation ($P = 0.0003$), i.e. they showed different allometric trends. Visually, differences in allometric patterns of shape were apparent at both the non-uniform and uniform components (Fig. 4).

3.4. Allometry-free differences

A common principal components model was verified for two sets of five measurements: W12, W23, W34, W26, W15, and W12, W23, W45, W56, W26. It was not rejected in females ($P = 0.17$) and in males ($P = 0.47$), as well in the total sample ($P = 0.32$). For males, females, and for the total sample (Fig. 5), canonical variate analyses were highly significant and produced a perfect reclassification of individuals (Table 5). This indicates consistent differences between *R. robustus* and *R. prolixus*, which could not be explained by their size difference.

Table 4
MANCOVA

Var dep (Y)	x1, y1, x2, y2, x3, y3, uniX, uniY
Var indep (X)	sp, ctr, sp*ctr
Wilk's statistics:	
sp	$P < 0.0001$
ctr	$P < 0.0001$
sp*ctr	$P = 0.0003$

This multivariate analysis of covariance (MANCOVA) indicates different allometries for *R. prolixus* and for *R. robustus*. Var dep (Y), dependent variable; Var indep (X), independent variables; sp, species; ctr, centroid size; sp*ctr, interaction between species and centroid size; x1, xy1, x2, y2, x3, y3, variables depicting the non-uniform component of shape; uniX and uniY, variables describing the uniform component of shape.
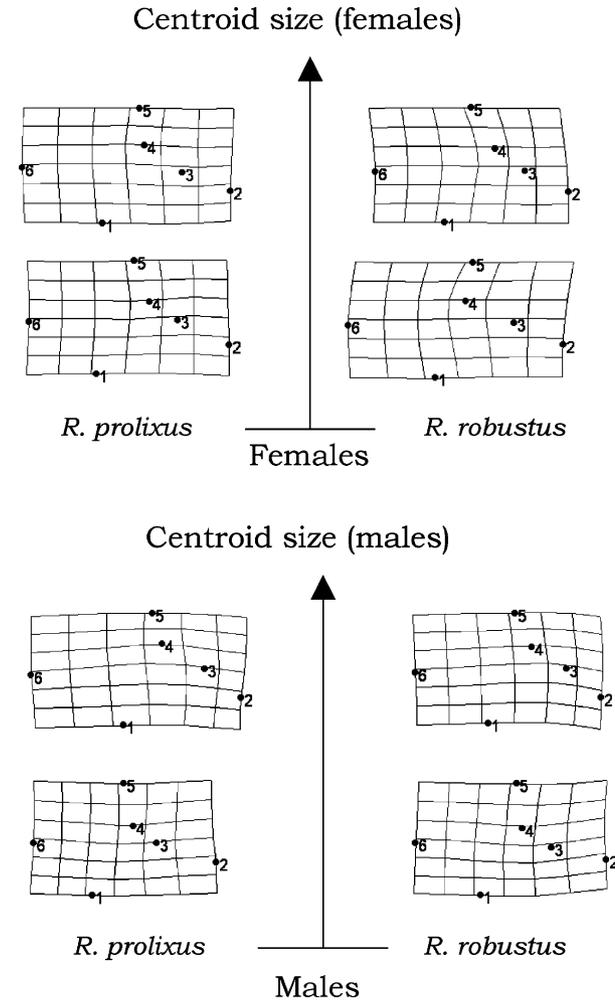

Fig. 4. Allometries. A consistent uniform change (stretching of the whole grid) is apparent in female *R. robustus*, another one in male *R. prolixus*, while no such change is visible in the corresponding sex of the other species. Non-uniform changes are also apparent, involving landmarks 2–5: larger *R. robustus* tend to have these homologous points more or less aligned, especially in females.

4. Discussion

R. prolixus and *R. robustus* have been compared several times using different techniques and different samples. Their status as distinct species has been questioned, leading to the idea that they may represent a single species able to freely alternate between sylvatic and domestic ecotopes. The present data do not fit with this hypothesis.

4.1. Size variation

R. robustus wings were significantly larger than those of *R. prolixus*, and allow clear differentiation between the two taxa. For the hypothesis of a single species, this size variation accords with the observation that sylvatic specimens of Triatominae (e.g. in palm trees) generally show larger size than their domestic counterparts (Dujardin et al., 1999b).
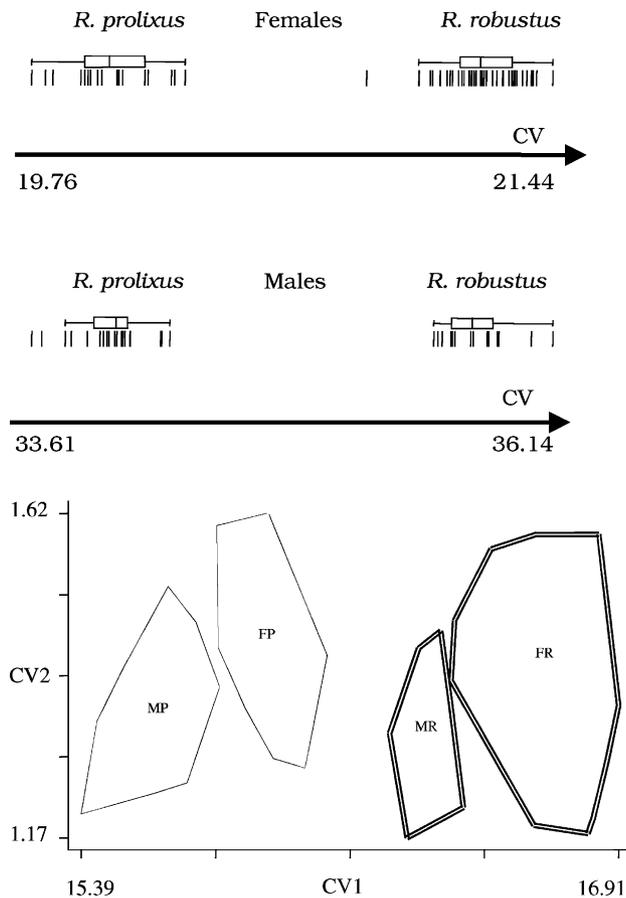
Fig. 5. Allometry-free discrimination. Top and middle: quantile plots of female (top) and male (mid) specimens belonging to *R. prolixus* and *R. robustus* along the discriminant factor (CV) derived from all the common principal components except the first one. Each box shows the group median separating the twentieth and seventy-fifth quartiles, with the tenth and ninetieth quartiles shown as lines on the right and left sides of the box. Bottom: factorial map presenting the distribution of *R. prolixus* (P) and *R. robustus* (R), male (M) and female (F) specimens, in the plane of the first two canonical factors (CV1 and CV2). Polygons enclose each group. Male and female *R. robustus* are double lined polygons, male and female *R. prolixus* are single lined polygons.

However, it disagreed with Bergmann's rule predicting larger specimens at lower temperatures (Table 1)–a rule that has been applied to the taxonomy of other insects such as phlebotomine sand flies (Marcondes et al., 1998; Dujardin et al., 1999a).

4.2. Shape variation

Shape variation, as described by geometric techniques, revealed significant but incomplete differences between taxa (Fig. 3, Table 3). For the hypothesis of one group being a larger or smaller representative of the other, it was expected that allometric changes alone would be responsible for these differences. Multiple regression of shape on size revealed a significant allometric content (36%), justifying both a comparison of the allometric trends and a tentative to remove them from the observed variation.

Table 5
Allometry-free discrimination

Initial variables	W12, W23, W34, W26 and W15 or W12, W23, W45, W26 and W56		
Shape variables	cpc2, cpc3, cpc4, cpc5		
	prolixus	*robustus*	Total, predicted
Females			
prolixus	18 (100%)	0	18
robustus	0	48 (100%)	48
Total (observed)	18	48	66
Males			
prolixus	20 (100%)	0	20
robustus	0	16 (100%)	16
Total (observed)	20	16	36

Re-classification of individuals according to discriminant analyses on allometry-free variables. W12, W23, W34, W45, W26, W56, W15, "initial" metric characters (for instance W12 is the distance measured between landmarks W1 and W2, see Fig. 1); cpc2, cpc3, cpc4 and cpc5, the five last common principal components used as allometry-free variables (see text), which are derived from the "initial variables". Initial variables are two sets of 5 distance measurements (either W12, W23, W34, W26, W15 or W12, W23, W45, W26, W56) for which the model of a common allometric axis could not be rejected (see text). In brackets, percent of correct attributions.

4.3. Divergent allometric trends

The within-taxa allometric trends of the wings were explored by geometric morphometrics, and showed consistent differences, as confirmed statistically by a multivariate analysis of covariance (Table 4), and illustrated by the thin-plate splines (Fig. 4). Such divergence, although commonly found between distinct species, might also develop within a single species occupying different habitats. It then would depend upon some exogenous factor varying across habitats and inducing altered patterns of local regulation during growth, or differences in the timing of specific developmental events (heterochrony), or some combination of both (Bookstein et al., 1985). But whatever the original cause, the consistent allometric differences after one generation in similar environmental conditions would seem to reflect stable genetic differences acquired after prolonged adaptation to different ecotopes. This is hardly compatible with common exchanges of specimens between habitats.

4.4. Allometry-free differences

Neither the total set of variables nor the seven possible subsets of six of them were compatible with the model of a single allometric axis common to both *R. robustus* and *R. prolixus*. And out of the 21 possible subsets of five characters, only two were compatible with a common axis. Using variables describing the orthogonal variation to this axis–i.e. all the CPC except the first one (Klingenberg, 1996), significant differences were apparent with both sets of measurements (Fig. 5, Table 5).

Such patterns of size-free variation indicate that our samples did not fit with the hypothesis of the two groups being the allometric extensions of each other. Between conspecific populations, it is expected that any shape differences (allometry-free) would be moderate or insignificant (Houck et al., 1990). Moreover, such differences between two sympatric populations would be good evidence for reproductive isolation (Claridge and Gillham, 1992). Nevertheless, shape differences also arise among conspecific populations, for example on both sides of a consistent ecological or geographical barrier (Burnaby, 1966; Dujardin et al., 1999a) or among relict populations from past geological events (Baylac and Daufresne, 1996).

We believe that distinct sizes, dissimilar allometric trends and allometry-free shape divergence could result from a variety of sources, including adaptation to separate ecotopes–*R. robustus* inhabits palm trees at Mérida, and *R. prolixus* infests dwellings at Cojedes. Since these effects could not be removed after one generation in the same environmental conditions, they are suggestive of true evolutionary divergence, and so they question any suspicion of a single species.

This biological question is of strategic importance, because in case of separate taxa, the sylvatic species (*R. robustus*) is not expected to replace the domestic one (*R. prolixus*) after insecticide application on domestic structures. Since our data were not compatible with the hypothesis of free exchange between sylvatic and domestic habitats, they allow to consider *R. robustus* and *R. prolixus* as separate targets for control strategies. Our study covered a limited geographic range, and it would be appropriate to examine further populations of *Rhodnius* from palm trees in Venezuela.

The methodology described here could also be applied to other insects of medical importance.

Acknowledgements

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