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Host alkaloids differentially affect developmental stability and wing vein canalization in cactophilic *Drosophila buzzatii*

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venation pattern.

Abstract

Host shifts cause drastic consequences on fitness in cactophilic species of *Drosophila*. It has been argued that changes in the nutritional values accompanying host shifts may elicit these fitness responses, but they may also reflect the presence of potentially toxic secondary compounds that affect resource quality. Recent studies reported that alkaloids extracted from the columnar cactus *Trichocereus terscheckii* are toxic for the developing larvae of *Drosophila buzzatii*. In this study, we tested the effect of artificial diets including increasing doses of host alkaloids on developmental stability and wing morphology in *D. buzzatii*. We found that alkaloids disrupt normal wing venation patterning and affect viability, wing size and fluctuating asymmetry, suggesting the involvement of stress–response mechanisms. Theoretical implications are discussed in the context of developmental stability, stress, fitness and their relationship with robustness, canalization and phenotypic plasticity.

Introduction

Chemical stress imposed by host plants is presumably a key a factor determining diversity, abundance and distribution of phytophagous insects (Schoonhoven *et al.*, 2005). In fact, stress or its counterpart, the buffering mechanisms, might be important selection targets during the evolutionary process of adaptation, especially when populations are colonizing marginal or new environments. However, little is known about the underlying processes that lead to stress resistance. For instance, qualitative and quantitative aspects of stress have been shown to be highly variable across species, suggesting that stress is an attribute not only of the stressor but also of the stressed (Badyaev, 2005; Bijlsma & Loeschcke, 2005). In addition, evolution of adaptive traits may result in correlated responses and trade-offs that might determine particular evolutionary trajectories, which are expected to be genotype and/or environment specific (Bijlsma & Loeschcke, 2005). Even more, stress could be acting as a major factor in marginal habitats, driving adaptive evolution by selecting robust genotypes and thus shaping population genetic structure with possible implications in the speciation process (Bijlsma & Loeschcke, 1997). Even though phenotypic robustness is expected to enable species to face stressful environments until establishment, also plasticity is expected to enable physiological tolerance avoiding extreme specialization in the resource under exploitation (Parsons, 1982).

In this regard, the cactus–*Drosophila* system is a well-recognized model to investigate the role of the use of host plants in the evolution of adaptive traits and the role of plant-specific chemistry in the process (Fogleman & Danielson, 2001; Soto *et al.*, 2014). The effects of host shifts on life history and morphology are well documented in several cactophilic species of *Drosophila* (Soto *et al.*, 2008; Matzkin, 2012; Soto *et al.*, 2014). One of the primary factors determining patterns of host plant specificity in the *Drosophila*–cactus system is the presence of secondary chemical compounds such as alkaloids that are potentially toxic for the flies (Fogleman & Danielson, 2001). Although more than 20% of angiosperms produce alkaloids (Schoonhoven *et al.*,

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2005), few empirical works have studied the effect of these natural defences on insect development.

Drosophila buzzatii is a cactophilic species of the buzzatii complex (repleta group) that inhabits the arid and semiarid lands of southern South America. Although prickly pears (genus *Opuntia*) are its main natural hosts, *D. buzzatii* can exploit columnar cacti of the genera *Trichocereus* and *Cereus* as secondary hosts (Barker, 1982; Soto *et al.*, 2008). Recent studies suggest the presence of alkaloids as the main chemical difference between *O. sulphurea* and *Trichocereus terschekii* (Hasson *et al.*, 2009; Padró & Soto, 2013; Carreira *et al.*, 2014), two of the most important *D. buzzatii* host plants in Argentina. Actually, *T. terscheckii* is especially rich in phenethylamines alkaloids (Reti & Castrillon, 1951; Corio *et al.*, 2013), and recent studies showed that an alkaloid fraction causes detrimental effects by impairing viability and decreasing body size (Corio *et al.*, 2013). These results are consistent with the idea that alkaloids are part of antiherbivory defence mechanisms to which insects must adapt to successfully exploit the resource (Wink, 2006). In addition, alkaloids' concentrations are highly variable among *Trichocereus* species, depending on the environment, individuals, organs and seasons (Reti & Castrillon, 1951; Wink, 2006; Ogunbodede *et al.*, 2010). In fact, *T. terscheckii* alkaloid concentration can vary from 0.25 to 1.5% of plant dry weight (Reti & Castrillon, 1951), indicating that this host plant may be regarded as an unpredictable and variable (even extreme) stressor, making it a potential marginal habitat for *D. buzzatii*.

Although metabolic pathways affected by alkaloids remain unclear, it is known that ingestion of alkaloid-rich food during larval life causes a general fitness decline (Soto *et al.*, 2014). Such general fitness decline suggests that flies reared in the presence of alkaloids experience stressful conditions during metamorphosis that should have detectable morphological consequences (Narberhaus *et al.*, 2005; Corio *et al.*, 2013). One way of detecting such stress is by assessing levels of fluctuating asymmetry (FA) in bilateral organs (Markow, 1995). FA reflects the degree of within-individual bilateral variation as consequence of random perturbations accumulated during development (Palmer & Strobeck, 1986). FA could be modulated by both extrinsic (environmental stress) and intrinsic (corrective buffer mechanisms) causation sources. Thus, FA may be considered as a measure of the ability of individuals to buffer the effects of perturbations during development and, thus, a trait that may be linked to fitness (Palmer, 1994). Nevertheless, the use of FA as a universal environmental predictor is a controversial topic in the literature, as some authors have found a positive correlation between stress and FA (McKenzie & Yen, 1995; Allenbach *et al.*, 1999), whereas others found no association (Rabitsch, 1997; Bourguet *et al.*, 2004). This scenario has led to the discussion on whether environmental stressors may or may not affect FA, and what are their causes (Palmer, 1994; Markow, 1995; Rasmuson, 2002; Lens *et al.*, 2002; Leamy & Klingenberg, 2005; Dongen, 2006). In this regard, more recently, FA studies have been shifting towards a more comprehensive framework taking account bias factors, which might mask the interaction between FA, fitness and stress (Floate & Fox, 2000; Polak *et al.*, 2002; Stamenkovic-Radak *et al.*, 2000).

In this work, we study the reaction norm of fitness components and FA to increasing doses of alkaloids, by incorporating threshold models, to test whether and to which extent the effect becomes detectable. We focus on wing morphology as its development is well known and has been extensively studied in many species exposed to different sources of environmental variation, and also, much of its genetic basis has been fully described. Also, wing size is considered a fitness predictor as it correlates with reproductive success, longevity, fertility and tolerance to extreme temperatures (Roff, 2000 and references therein).

Materials and methods

Fly stocks and alkaloids extraction

Fly stocks of *D. buzzatii* used in this work were derived from gravid females collected in the locality of San Agustín del Valle Fértil (30°38′4″S, 67°28′6″W, northwestern Argentina) and maintained for several generations as iso-female lines under controlled conditions until the onset of the experiments described below.

Pieces of fresh tissues of *T. terscheckii* were also collected for alkaloid extraction, which was carried out by partitioning a concentrated EtOH extract with diluted HCL acid to pH 3 and extracting with CH_2CL_2. Aqueous layer was alkalinized to pH 12 with sodium hydroxide (pKa mescaline, 9.5) and extracted three times with CH_2CL_2. The crude alkaloid fraction yield was 0.33 mg per gram of fresh tissue (4.5 mg g^{-1} of dry weight). Alkaloids' identification was performed by gas chromatography–mass spectrometry (GC-MS) showing the typical mescaline, N-dimethylmescaline and α-methylmescaline known to be present as major components in the tissues of this species (Corio *et al.*, 2013 for details).

Experimental treatments

Iso-female lines were outbred for three generations before the experiments. Batches of 30 first instar larvae were seeded in vials containing Instant *Drosophila* Medium (Carolina Biological Supplies) plus varying quantities of the alkaloid fraction extracted from *T. terscheckii* (see below). Vials were incubated under a 12:12 h light/dark photoperiod at 25 ± 1 °C until adult emergence.

The design included ten replicates for each of the following treatments: C) control: vials contained 1 g

of standard laboratory rearing medium; A1) vials containing 1 g of standard laboratory medium plus the same quantity of the alkaloid fraction found in 1 g of cactus (4.5 mg g^{-1} of dry weight); A2) similar to A1 but with a final concentration of the alkaloid fraction 50% higher (6.75 mg g^{-1} of dry weight); and A3) alkaloid fraction concentration was twice the concentration in A1 vials (9 mg g^{-1} of dry weight).

Performance assessment

Viability was measured as the proportion of adults emerged in each vial relative to the number of larvae seeded. The resulting data were analysed by means of pairwise comparisons between each treatment mean and the control (Dunnett's tests). Data were angularly transformed to comply with the assumptions of the test (Zar, 1996). Viability scores for this experimental set up along with a description of wing morphology for a subset of individuals were previously reported elsewhere (Corio *et al.*, 2013). In this study, we use those values as a fitness proxy, but performed new analyses with normal and abnormal wings that complied the requirements for the subsequent morphological analyses. For this, both wings from each fly were removed and mounted on slides with DPX (Sigma Aldrich, St. Louis, MO, USA) and photographed at 20× magnification using a Leica S4E stereo microscope. Thus, a total of 175 pairs of wings (81 males and 94 females) were included in the analysis by positioning the most representative landmarks at the junctions of the most representative veins and wing margins (Fig. 1). This procedure was performed twice by the same person to contemplate the experimental error in the analyses (Individual error factor). Wing size was studied separately from shape variation using geometric morphometric techniques. Wing size was estimated via the centroid size of each individual configuration of landmarks, whereas wing shape variation was computed by means of least squares Procrustes superimposition method, to examine differences in the landmarks position (Mardia *et al.*, 2000).

A two-way ANOVA was employed to investigate wing size variation with 'Treatment' and 'Sex' and its interaction as fixed factors. The residual variance was assessed by means of the average of individual measurements.

We also performed a segmented regression analysis to test the often assumed linear no-threshold model (Parson, 2003), by incorporating break-point models. This approach compares simple linear regressions with segmented regressions, estimating the concentration range giving the maximal effect and evaluating the model that best describes the data. Thus, we performed a segmented linear regression weighted by sample size using SegReg V.2013 software (Oosterbaan *et al.*, 1990) to determine the existence of a break point. Piecewise regression model with two straight lines at the break point results in the following equations:

$$Y_i \begin{cases} \beta_0 + \beta_1 \chi_i + e_i & \text{For} \chi_i \leq \alpha \\ \beta_0 + \beta_1 \chi_i + \beta_2 (\chi_i - \alpha) + e_i & \text{For} \chi_i \leq \alpha \end{cases}$$

where Yi denotes the value of *i*th observation, χ_i corresponds to the independent variable value and e_i is the error. The threshold is represented by α (break point). The slopes of the lines are β_1 and $\beta_1 + \beta_2$; thus, β_2 can be considered as the difference in slopes (Toms & Lesperance, 2003).

We further investigated the effect of alkaloids on the cellular basis of wing size by scoring cell size and cell number (Azevedo *et al.*, 2002). To this end, the distal-posterior wing compartment (between LV4 and LV5; Fig. 1) was photographed at 400× magnification using a NIKON E200, Tokyo, Japan compound microscope. Each trichome within 100 μm² (calculated using Olympus 0.01 mm scale) of the epidermal cell area was counted (Fig. 1a,b) using ImageJ (version1.46r), and cell area was calculated by dividing 100 μm² by trichome count. Variation in cell area between sexes and treatments was evaluated with an ANOVA. To detect differences in cells number, we calculated a total cells' number index by dividing wing area by cell area. The resulting index was used as the dependent variable in an ANOVA with 'Sex' and 'Treatment' and its interaction as fixed factors.

Asymmetry variation

For size FA, departures from bilateral asymmetry were independently studied in each treatment by partitioning phenotypic variation into among individuals, within individuals and error components, using the centroid size in a two-way ANOVA with factors 'Individual' and 'Side' and their interaction 'Individual' x 'Side' (details in: Palmer & Strobeck, 1986; Palmer, 1994). The dependence of asymmetry with size was assessed twice by performing Pearson correlations between individual wing size (average measurement ($R_s + L_s/2$)) and FA1 and FA2 fluctuating asymmetry indexes within each treatment. FA1 was calculated as the scale index (average measurement (abs(R_s-L_s))), whereas FA2 was calculated as the unscale index (FA1/($R_s + L_s/2$) for each individual. Additional FA10 index was also calculated to investigate the fluctuant asymmetry pattern after removing measurement error (Palmer & Strobeck, 1986; Palmer, 1994). A two-way ANOVA with FA1 index was performed with 'Treatment' and 'Sex' and its interaction to evaluate the sex dimorphism effect.

To determine whether size FA varied as a function of the chemical stress imposed by the presence of alkaloids (Palmer & Strobeck, 1986, pp. 409–410), the same segmented regression model design as for centroid size was applied.

Asymmetry on wing shape was analysed using Procrustes ANOVA method (Klingenberg & McIntyre, 1998),

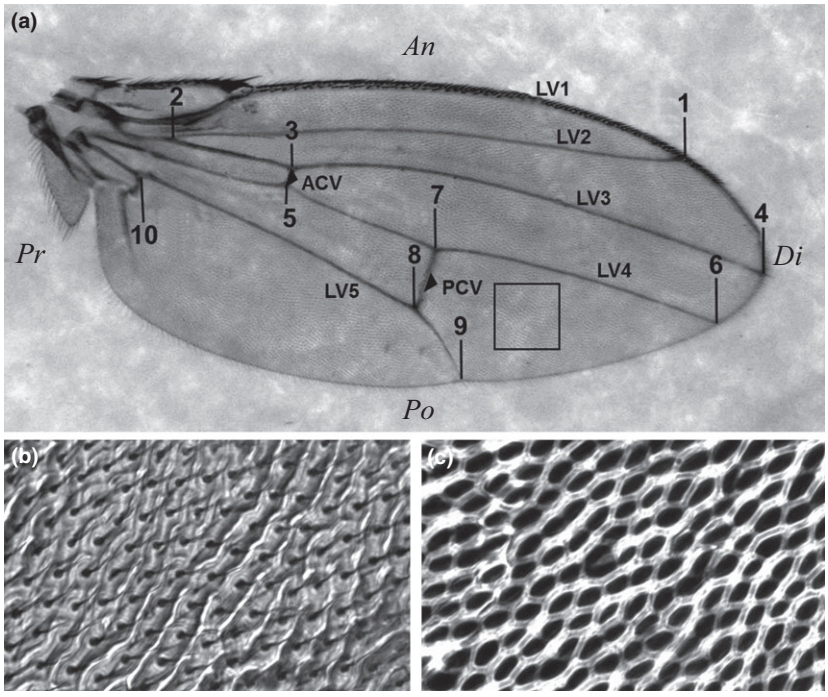

Fig. 1 *Drosophila buzzatii* wing. (a) Landmark positioning and wing references. *An*: Anterior; *Po*: Posterior; *Pr*: Proximal; *Di*: Distal; L: Longitudinal vein; ACV: Anterior cross-vein; PCV: Posterior cross-vein. The square shows the location of trichome counts. High magnification images (400× Phase contrast microscopy) of the counting region in the posterior compartment of wings shows the trichome *per* cell expression (b) and cell area delimited by extracellular matrix (c).

and *P*-values were obtained by 10 000 permutations as implemented in SAGE software (Marquez, 2006).

Means squares of 'Individual x Side' factor were used to compare FA levels among treatments using standard *F*-tests (Debat *et al.*, 2009) with further Holm–Bonferroni correction.

Shape variation

A number of anomalies in wing venation pattern were detected in the initial inspection of the slides (Fig. 2). Chi-square contingency tests were used to analyse the effect of treatments and sex on the incidence of anomalies. The data set was arranged in a 2 × 4 table with 'Anomaly' (# of cases) and 'Treatment' (four levels) as factors. We also arranged the data set in a 2 × 2 table with 'Sex' and 'Anomaly' as factors to test whether anomalies were randomly distributed among sexes. For this analysis, we perform the Cochran–Mantel–Haenszel test (CMH), which allows the comparison of two groups on stratified categorical data (Wittes & Wallenstein, 1993). Finally, as anomalies could be easily classified into three main groups (Fig. 2), a new data set was created using the incidence of each type of anomaly. Thus, data were arranged in a 3 × 3 table to test whether the frequency of anomaly types was correlated to alkaloids' concentration. All analyses were performed using Infostat (Di Rienzo *et al.*, 2013).

As some anomalies compromised homology in landmarks positioning, we decided *a posteriori* to exclude landmark N°8 (in the junction between LV5 and PCV; Fig. 1) and study the quantitative effect of the 'Treatment' and 'Sex' in the rest of the wing landmarks. The analysis on wing shape was performed by means of a MANCOVA with 'Treatment', 'Sex' and the interaction 'Treatment by Sex', with centroid size as covariate. Dependent variables were extracted from the principal component analysis (PCA) scores, which can be seen as orthogonal features of shape variation. For the MANCOVA, we used the first seven PC scores that ensured covering at least 95% of the total variance while excluding measurement error often found in the lasts PCs (Breno *et al.*, 2011). To further investigate the 'Treatment by sex' interaction, a canonical variate analysis (CVA) was performed by testing pairwise comparison between 'Sex by Treatment' using Procrustes distances (10 000 permutation; MorphoJ software; Klingenberg 2011).

Variation patterns

To test differences in the canalization pattern across treatments, the mean squares corresponding to 'Individual' factor in the ANOVA s were used as an estimator of among-individual variation (size and shape VAR; Debat *et al.*, 2001, 2009) and standard F-tests were performed between treatments.

For testing the link between canalization (among-individual variation) and developmental stability (within-individual variation), different estimators were calculated for size and shape (Debat *et al.*, 2009; Breno *et al.*, 2011, 2013). For size, Manhattan and Euclidean distance (standardized) matrices were computed using the individual centroid size (average $(R_s + L_s)/2$).

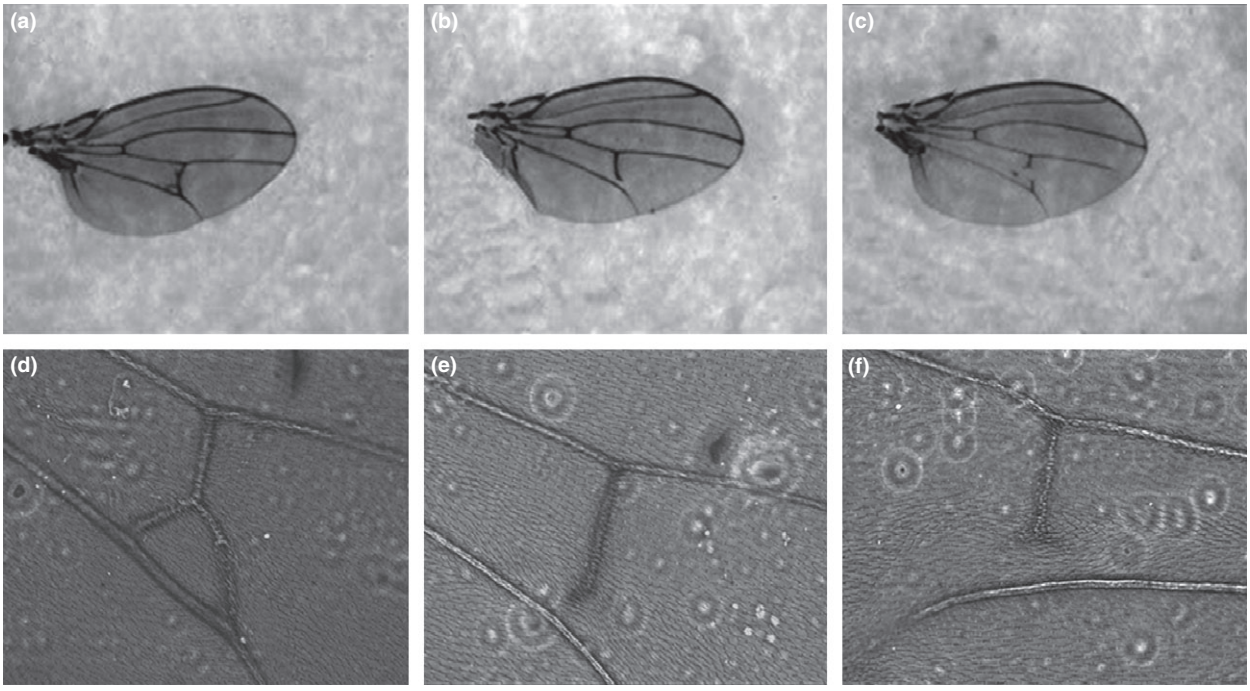

Fig. 2 Types of anomalies in the wing venation pattern involving the posterior cross-vein found in flies reared in vials with alkaloid concentrations. (a) Bifurcation of the posterior cross-vein (PCV), (b) nonjunction of the PCV and the longitudinal vein (L5) and (c) a combination of both anomalies in the same wing. (d), (e) and (f) magnification of the affected area of each anomaly type.

Among-individual variation estimators for each individual was constructed by summing the columns of the matrices (distance of each individual to all others), and further, Pearson correlation analyses were run with its corresponding FA1 (within-individual variation). For shape, Mantel matrix correlation tests between the variance–covariance matrix of 'Individual' (among-individual variation) vs. 'Side x Individual' (FA) for each treatment were performed with 10 000 random permutations (the diagonal blocks of the covariance matrix were excluded to avoid spuriously high correlations).

In addition, the observation of conspicuous phenotypic differences between individuals (noted as wing vein abnormalities) allowed us to cluster them into two categories. Thus, we could perform a more direct test to evaluate FA differences between canalized and decanalysed phenotypes (normal vs. abnormal wing vein pattern). To this purpose, the same stated FA analysis for size (two-Way ANOVA) and shape (Procrustes ANOVA) was calculated independently for both groups to include their respective mean squares for further *F*-tests.

Results

Dunnett's test for viability between the control (mean: 0.48 ± 0.03) and alkaloid treatments revealed that the two higher concentrations (A2 mean: 0.35 ± 0.04; A3 mean: 0.14 ± 0.03) were significantly lower, whereas A1 treatment (mean: 0.44 ± 0.03) did not differ from the control (Dunnett's tests: $F_{3,36} = 20.15$, $MS = 0.16$, $P = 0.04$, $P < 0.01$ and $P = 0.75$, respectively).

The general ANOVA testing for differences in wing size revealed significant 'Treatment' and 'Sex' (females were 9% larger than males) and 'Sex by Treatment' effects ($F_{3,167} = 16.53$, $P < 0.001$; $F_{1,167} = 321.99$, $P < 0.001$; $F_{3,167} = 5.40$, $P < 0.01$; Table 1).

Due to significant interaction between 'Sex' and 'Treatment', segmented regression analyses for size were performed independently. The test revealed a biphasic function determined by an action threshold close to the lowest alkaloid concentration (Fig. 3a) for both females ($\alpha = 1.32 \pm 0.1$, $F_{4,89} = 13.74$, $P < 0.001$) and males ($\alpha = 0.99 \pm 0.1$, $F_{3,77} = 5.21$, $P < 0.001$), with positive slopes at concentrations below the break point (males: $\beta = 0.1$, $r^2 = 0.01$; females: $\beta = 0.43$, $r^2 = 0.18$) and negative slopes beyond this point (males: $\beta = -0.3$, $r^2 = 0.18$; females: $\beta = -0.8$, $r^2 = 0.33$; additional results in Table A1, Appendix).

Cellular basis of size variation analysed by ANOVA s revealed that for cell area, 'Sex' and 'Treatment' factors were significant, although its interaction was not ($F_{3,167} = 3.56$, $P = 0.01$; $F_{1,167} = 95.17$, $P < 0.001$; $F_{3,167} = 0.11$, $P = 0.95$; Table 1). Females' cell area was, in average, 13.3% bigger than in males, whereas the 'Treatment' effect revealed a 6.4% average decline in A3 compared to A1 and 5% with respect to the control.

Table 1 ANOVAS testing for differences in wing size (centroid size), cell area and cell number among treatments, between sexes and its interaction.

Variable	Factor	d.f	MS	*F*
Wing Size	Treatment	3	3.79	16.53***
	Sex	1	73.90	321.99***
	Sex × Treatment	3	1.23	5.40**
	Error	167	0.22	
Cell area	Treatment	3	1.59	3.56*
	Sex	1	42.54	95.17***
	Sex × Treatment	3	0.01	0.11
	Error	167	0.44	
Cell number	Treatment	3	1.25	0.68
	Sex	1	17.11	9.28**
	Sex × Treatment	3	1.48	0.80
	Error	167	1.84	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Results regarding cell number analyses showed that only the 'Sex' factor was significant ($F_{3,167} = 0.68$, $P = 0.57$; $F_{1,167} = 9.28$ $P < 0.01$; $F_{3,167} = 0.80$, $P = 0.5$; Table 1). In this sense, females' cell number was, in average, 5% larger than in males. Thus, the cellular basis of sexual dimorphism was caused by both cell area and cell number, whereas wing size differences among treatments were mainly caused as a result of cell size variation (mean values in Table A2, Appendix).

Wing Asymmetry

Before analysing the effect of increasing doses of alkaloids on FA levels, preliminary analyses for testing significant fluctuating asymmetry were performed by separating FA from directional asymmetry and measurement error. The ANOVA s showed the absence of directional asymmetry ('Side' factor) in all cases ($P > 0.05$). In addition, Shapiro–Wilks and Lilliefors tests for the differences between sides did not reveal any significant departure from normality (^{W}Control = 0.95, $P = 0.05$; ^{W}A1 = 0.96, $P = 0.07$; ^{W}A2 = 0.97, $P = 0.11$; ^{W}A3 = 0.96, $P = 0.46$). Variance among individuals ('Individual' factor) explained a significant proportion of overall variation in all experimental groups ($P < 0.001$). Furthermore, significant levels of fluctuating asymmetry ('Side × Individual' interaction) with respect to the measurement error were detected in all treatments ($P < 0.001$), which enables further tests (Table 2).

To test size FA differences among treatments, an evaluation on asymmetry-size dependence was performed. As both rearing medium and sex affect wing size (Table 1), Pearson correlation analyses between individual FA indexes and wing size values were run for each treatment. All correlation analyses between individual FA indexes and size were not significant ($P > 0.05$), whereas FA indexes were shown to be highly correlated between each other (RC = 0.99, $P < 0.0001$). Thus, we decided to investigate size FA differences among treatments using the rawer FA1 index (unscale) as the dependent variable in the ANOVA (mean indexes by treatment in Table A3, Appendix). Given that the only significant factor was 'Treatment' ($F_{3,167} = 3.03$, $P = 0.03$; $F_{1,167} = 0.49$, $P = 0.48$; $F_{3,167} = 0.33$, $P = 0.80$; Table 3), this analysis further confirmed that FA1 was not biased by sexual dimorphism in size. *Post hoc LSD Fisher's* tests revealed that the incidence of FA in A2 flies was significantly higher than in Control flies ($P < 0.004$) and that FA levels in A3 flies was significantly lower than A2 ($P < 0.04$). As sexual dimorphism between males and females did not differentially affect levels of asymmetry (nonsignificant interaction, Table 3), we decided to pool them together for further FA analysis.

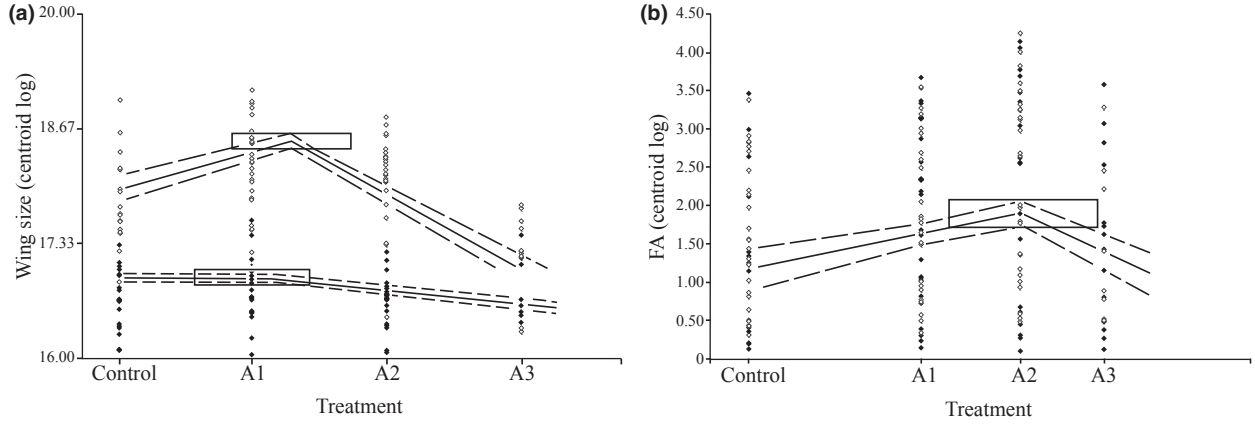

Fig. 3 Results of the segmented regression analyses for (a) wing size and (b) fluctuating asymmetry as a function of alkaloids concentration showing the best two-phase straight line regression describing the data. White and black circles represent females and males, respectively. 95% confidence intervals for regression band and the threshold (break-point box) are shown.

Table 2 ANOVAS and Procrustes ANOVAS testing for wing size and wing shape directional and fluctuating asymmetry for each treatment. Effects: *R* for Random factor and *F* for Fixed factor.

			Control			A1			A2			A3		
Variable	Factor	Effects	d.f	MS	*F*	d.f	MS	*F*	d.f	MS	*F*	d.f	MS	*F*
Size	Individual	*R*	43	231.50	65.14***	51	357.11	88.73***	54	414.98	69.22***	23	149.24	51.88***
	Side	*F*	1	0.39	0.11	1	3.86	0.96	1	3.14	0.47	1	6.29	2.18
	Side × Ind	*R*	43	3.55	30.88***	51	4.02	37.80***	54	6.00	46.85***	23	2.87	39.74***
	Error	*R*	88	0.11		104	0.10		110	0.13		48	0.07	
Shape	Individual	*R*	602	10.65	6.34***	714	12.78	7.74***	756	15.63	5.08***	322	13.85	4.67***
	Side	*F*	14	3.51	2.09	14	3.11	1.88	14	4.11	1.33	14	3.62	1.22
	Side × Ind	*R*	602	1.68	6.74***	714	1.65	6.26***	756	3.08	10.45***	322	2.96	11.71***
	Error	*R*	1232	0.25		1456	0.26		1540	0.29		672	0.25	

***$P < 0.001$.

The results of pairwise *F*-tests comparisons applied to the MS of the 'Individual x Side' interaction (size FA) agreed with *post hoc* tests. However, after multiple tests' correction, the comparison of A2/A3 treatments was close to significance (A2/A3 $F_{54,23} = 2.10$, $P_{H\text{-}B} = 0.05$; A2/Control $F_{54,43} = 1.70$, $P_{H\text{-}B} = 0.03$), indicating that differences are subtle, yet the pattern is not a measurement error artefact (Fig. 4e).

The segmented regression analyses (using FA1) testing for a threshold dose revealed a biphasic function with an action threshold at the intermediate alkaloid concentration and located the break point close to the A2 concentration ($\alpha = 2.1 \pm 0.1$), with positive and negative slopes at concentrations below ($\beta = 0.58$, $r^2 = 0.05$) and above the break point, respectively ($\beta = -1.21$, $r^2 = 0.04$, $F_{4,170} = 2.01$, $P < 0.001$; Fig. 3b; Additional results in Table A4, Appendix).

Regarding shape FA differences among treatments, Procrustes pairwise *F*-tests were employed. The comparisons between alkaloid treatments and the control revealed significant FA levels in the two higher concentration of alkaloids, whereas the lower alkaloid concentration showed no discrepancy with respect to the Control (A1/Control $F_{714,602} = 0.98$, $P_{H\text{-}B} = 0.60$; A2/Control $F_{756,602} = 1.83$, $P_{H\text{-}B} < 0.001$; A3/Control $F_{322,602} = 1.76$, $P_{H\text{-}B} < 0.001$; Fig. 4f).

Table 3 ANOVA testing for differences in asymmetry among treatments, sexes and the interaction (Treatment x Sex) using the FA1 index (size FA); the same effects were computed in a MANCOVA on the scores of a PCA (applied to the Procrustes coordinates), using as a covariate the centroid size.

	Effect		d.f	MS	*F*
Size FA	Treatment		3	44.05	3.03*
	Sex		1	7.08	0.49
	Treatment × Sex		3	4.76	0.33
	Error		167	14.51	
	Effect	**Wilks**	**d.f (num)**	**d.f (den)**	***F***
Shape	Treatment	0.96	21	962	0.63
	Sex	0.94	7	335	3.20**
	Size	0.94	7	335	3.27**
	Treatment × Sex	0.70	21	962	6.02***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Shape variation

In the preliminary morphological inspections, a number of abnormalities were found in wing venation pattern (Fig. 2). Their incidence was 12% in A1, 66% in A2 and 100% in A3, whereas no individuals with abnormal wing venations were detected in control vials (Fig. 4a).

In a qualitative analysis, a significant association between incidence of anomalies and alkaloid concentration was detected, either including or excluding the control (*Hoi/ii*, $P < 0.01$). However, correlation analysis excluding the control showed a linear trend ($P > 0.82$), whereas the trend departed from linearity when the control was included ($P < 0.05$). These tests also showed that anomalies were randomly distributed across sexes (*Hoiii*, $P = 0.83$). In addition, the frequency of anomaly types was not correlated to the alkaloids concentration (*Hoiv*, $P = 0.15$), and thus, the types of anomalies were also randomly distributed across alkaloid doses (Fig. 2, Table 4).

The quantitative analysis (excluding landmark N° 8) performed with the MANCOVA using PCA scores (eigenvalues presented in Table A5, Appendix) showed a significant shape wing sexual dimorphism beyond size effects (covariate) and a significant 'Sex' × 'Treatment' interaction ($F_{7,335} = 3.20$, W = 0.94, $P < 0.01$; $F_{7,335} = 3.27$, $W = 0.94$, $P < 0.01$; $F_{21,962} = 6.02$, W = 0.70, $P < 0.001$, respectively; Table 3). As both sexual dimorphism and treatments were found to affect wing shape, a canonical covariate analysis was performed to find the landmarks' displacements that best distinguish among these two factors. From the CVA applied to Procrustes distances between 'Sex by Treatment', individuals become clearly separated according to treatments

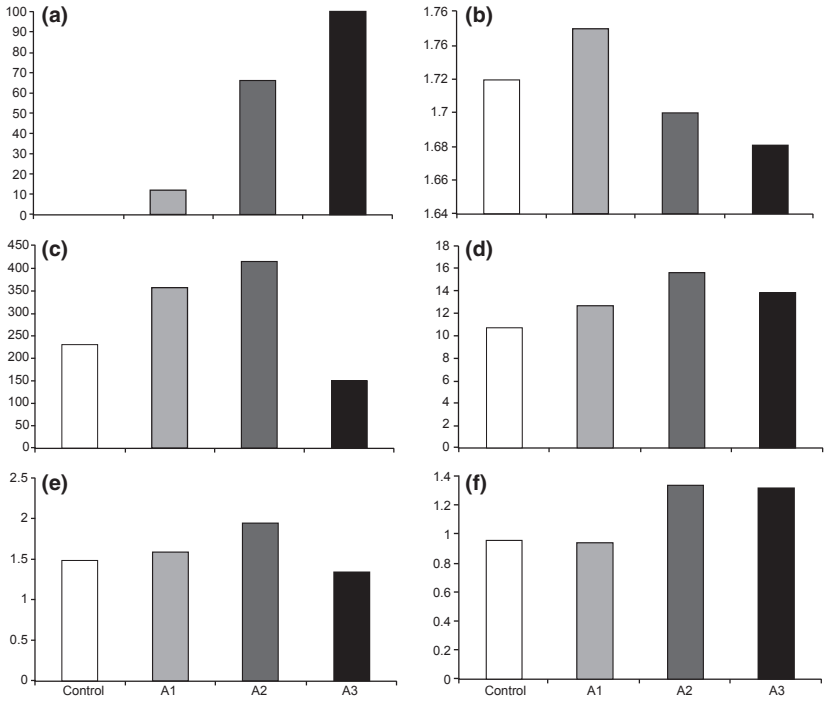

Fig. 4 Experimental treatments according to: (a) percentage abnormal phenotypes, (b) mean centroid size, (c) size VAR, (d) shape VAR, (e) size FA10 and (f) shape FA10.

Table 4 χ^2 contingency tests for hypothesis regarding the incidence of abnormalities in wing venation pattern.

	Total			Trend			Linearity deviation		
Hypotheses test	χ^2	d.f	*P*	χ^2	d.f	*P*	χ^2	d.f	*P*
H_{Oi}	199.72	3	< 0.01	193.82	1	< 0.05	13.02	2	< 0.05
H_{Oii}	114.57	2	< 0.01	118.47	1	< 0.05	0.05	1	>0.82
H_{Oiii}	0.04	1	0.83	(CMH)					
H_{Oiv}	6.62	4	0.15						

H_{Oi}: The incidence of anomalies and its trends does not differ between treatments; H_{Oii}: The alkaloid doses do not increase the incidence of anomalies when excluding the Control; H_{Oiii}: Anomalies are independent of sex; H_{Oiv}: Incidence of abnormalities types does not differ between alkaloid doses; CMH, Cochran–Mantel–Haenszel test.

along CV1 axis, which accounts for more than 80.8% of total variance (Fig. 5). The plot in the wireframe graphs along CV1 axis showed that as alkaloids' dose increases, the shape of the wing turns narrower and more elongated. Major changes resulting in this elongated shape involved the distal-proximal translation of landmarks N° 10 and N° 2, whereas the narrower shape was the result of the translation and rotation of landmarks N° 1 and 7 into a distal-posterior orientation, of landmark N° 4 into a proximal-posterior orientation and the translation of landmark N° 9 towards the anterior part of the wing. The CV2 axis clearly separated females and males; however, this axis accounted for only 10.6% of total variance. Some major changes in shape sexual dimorphism also involved elongated and narrow shapes, resulting from changes in the positions of some of the same landmarks as in CV1. However, narrower shapes along CV2 also involved the rotation and translation of landmarks N° 6 with an anterior-proximal orientation and N° 7 with an anterior-distal orientation (additional results in Table A6, Appendix).

Variation patterns

The results of association analysis between FA (within-individual variation) and among-individual variation across treatments differ in their patterns for size and shape. For size, *F*-tests applied to MS of 'Individual' factor (among-individual variation; Table 2; Fig. 4c) between alkaloid treatments and control showed

nonsignificant values after multiple test correction (A1/Control $F_{51,43} = 1.54$, $P_{H\text{-}B} = 0.34$; A2/Control $F_{54,43} = 1.79$, $P_{H\text{-}B} = 0.08$; A3/Control $F_{23,43} = 0.64$, $P_{H\text{-}B} > 0.99$). This indicates that variation among individuals did not increase as a function of the alkaloids' dose. Also, Spearman correlations between among-individual variation and their respective FA1 levels yielded no statistical significance for any treatment (all $P > 0.1$), suggesting a lack of relationship between patterns of variation across treatments.

In the case of wing shape, *F*-test on 'Individual' shape MS (Table 2; Fig. 4d) showed that among-individual variation was greater in all alkaloid concentrations compared with the control (A1/Control $F_{714,602} = 1.20$, $P_{H\text{-}B} = 0.01$; A2/Control $F_{756,602} = 1.47$, $P_{H\text{-}B} < 0.001$; A3/Control $F_{322,602} = 1.30$, $P_{H\text{-}B} = 0.01$). In addition, Mantel correlation analysis for the variance–covariance matrices of among-individual variation and FA was significant in all treatments (Control: $r^2 = 0.59$, $P < 0.0001$; A1: $r^2 = 0.58$, $P < 0.0001$; A2: $r^2 = 0.66$, $P < 0.0001$; A3: $r^2 = 0.46$, $P < 0.001$; Fig. 6).

Finally, *F*-tests for shape and size FA were performed between abnormal and normal wing vein patterns, as these two groups presented the most marked interindividual variation. *F*-test for size FA MS ('Ind x side'; Table 5) showed nonsignificant results between the two groups (Abnorm/Norm $F_{148,100} = 1.22$, $P = 0.14$), indicating a certain degree of independence between the incidence of abnormalities and FA. In contrasts, shape *F*-test showed that abnormal phenotypes tended to be more asymmetrical than normal phenotypes (Abnorm/Norm $F_{1022,1400} = 1.43$, $P < 0.001$).

Discussion

Stress in ecotoxicology is usually defined in terms of physiology, whereas in evolutionary biology, it is referred in terms of fitness. However, among ecologists, there is not agreement as some authors consider that changes in conditions that lead to a fitness decline may be considered as stress, whereas others point out that only conditions producing a marked fitness reduction should be considered as stressful (Hoffman & Woods, 2003). Viability is, along with fecundity, a main fitness component which is usually considered as an appraisal of the degree of adaptation of an organism's physiological and genetic mechanisms to exploit nutrients and eliminate toxic compounds (Soto *et al.*, 2014). In this regard, we found that viability depended on the alkaloids concentration to which larvae were exposed during development. Moreover, we included wing size (a proxy of body size) as another, though indirect, fitness indicator. Body size has traditionally been considered to be a key factor of the ecological and physiological

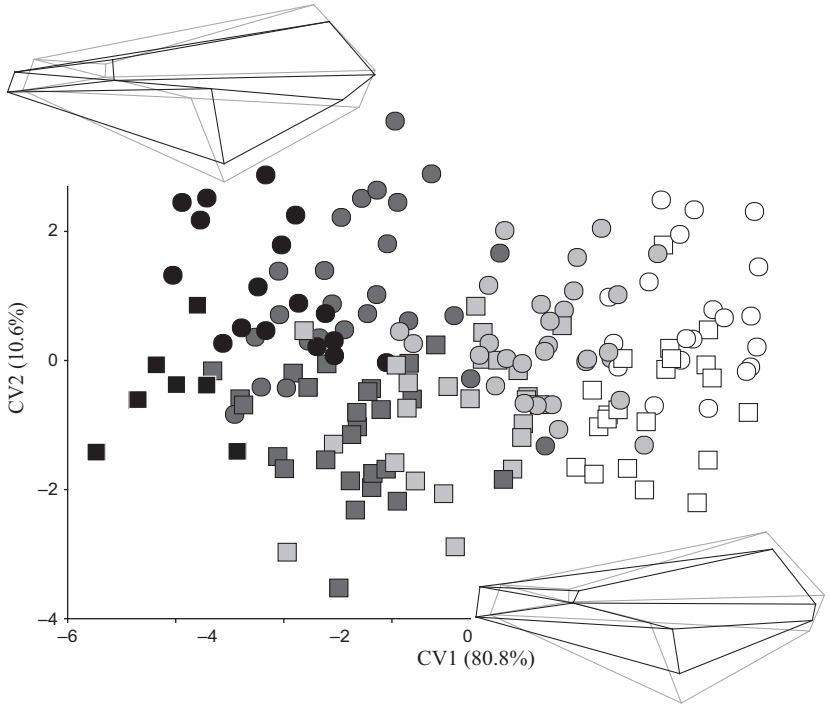

Fig. 5 Sex by Treatment interaction. First two canonical variate axes (and percentage of explained variance) are depicted. Squares and circles denotes males and females, respectively, whereas black stands for A3, dark grey for A2, light grey for A1 and white for Control treatment. Wing wireframe graphics display shape changes across the axes; black configurations denote extreme negatives values, whereas grey corresponds to positive values.

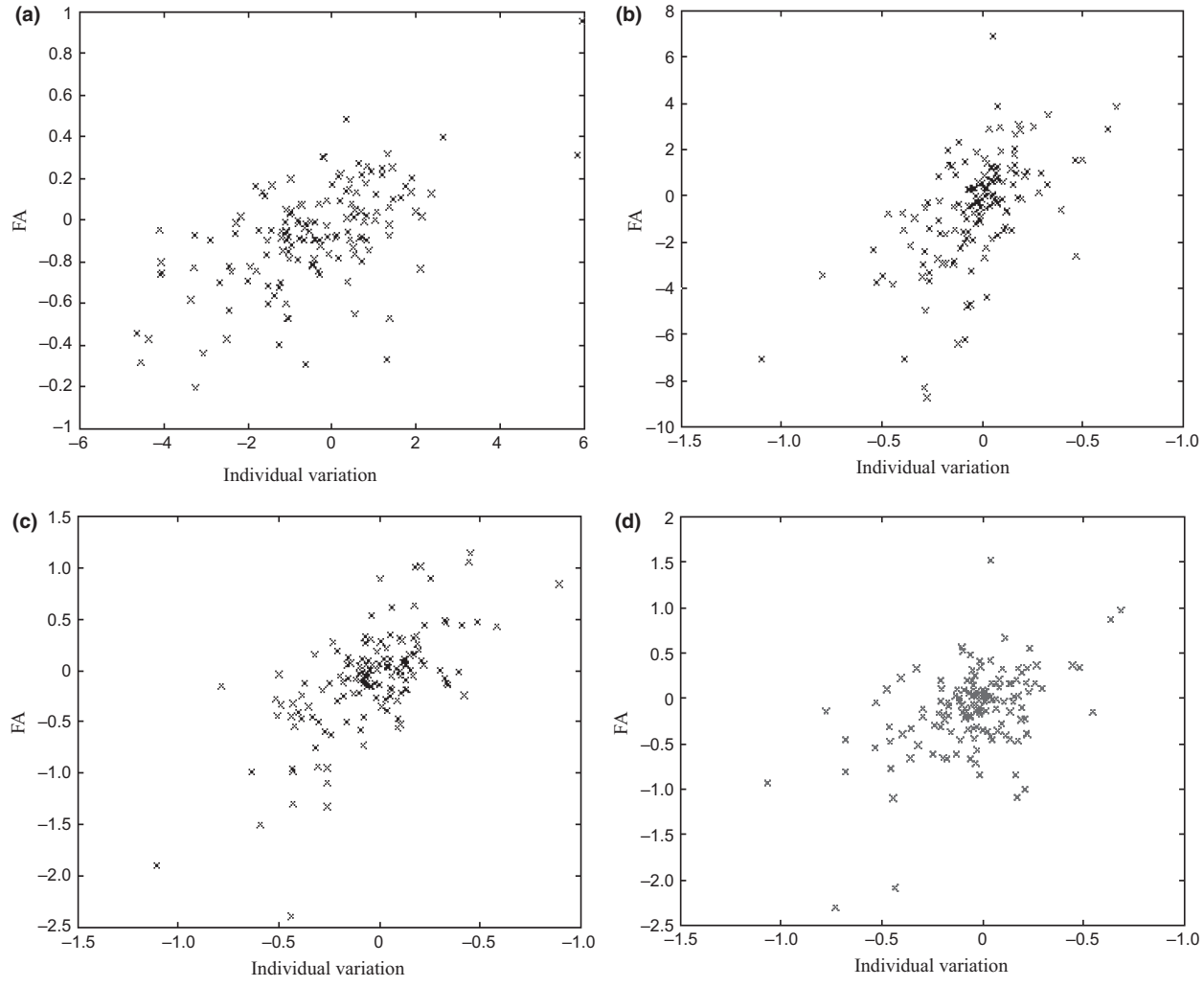

Fig. 6 Scatter plot of matrix correlations between shape FA and among-individual variation for the four treatments. (a) Control, (b) A1, (c) A2 and (d) A3.

Table 5 ANOVAS and Procrustes ANOVAS testing for wing size and wing shape directional and fluctuating asymmetry between phenotypes. Effects: *R* for Random factor and *F* for Fixed factor.

			Normal phenotypes			Abnormal phenotypes		
Variable	Factor	Effects	d.f	MS	*F*	d.f	MS	*F*
Size	Individual	*R*	100	296.61	74.79***	73	386.96	79.83***
	Side	*F*	1	4.60	1.16	173	6.63	1.36
	Side × Ind.	*R*	100	3.96	34.10***	148	4.84	47.00***
	Error	*R*	202	0.11			0.10	
Shape	Individual	*R*	1400	14.62	7.49***	1022	15.26	5.47***
	Side	*F*	14	3.68	1.89	14	2.58	0.92
	Side × Ind.	*R*	1400	1.95	7.42***	1022	2.79	10.13***
	Error	*R*	2828	0.26		2072	0.27	

***$P < 0.001$.

properties of organisms because it correlates with reproductive success, longevity, avoidance of predation, fecundity and tolerance to heat, cold and starvation among other traits (Roff, 2000 and references therein). Our results revealed that flies reared in vials with the highest alkaloid concentration attained smaller wing sizes paralleling and reinforcing the results observed for viability. These findings were expected as flies reared in *T. terschekii* have, on average, smaller wings than those reared in the more benign conditions offered by the primary *Opuntia* hosts (Soto *et al.*, 2008, 2014). Furthermore, we also showed that wing size decrease due to the presence of alkaloids in the rearing medium was mainly due to a reduction in cell area rather than cell number. It is known that environmental chemical stress can directly alter enzymatic, cellular and developmental processes, producing changes to the phenotype (Whitman & Agrawal, 2009). Moreover, toxic compounds are likely to exert their effect on enzymes involved in xenobiotics metabolism affecting individuals' metabolic costs by inducing detoxifying protein synthesis (Timbrel, 2009).

Our present results are also concordant with previous studies in other cactophilic *Drosophila*, showing that larvae reared in alkaloidiferous columnar cacti tend to produce flies with reduced size (Hurtado *et al.*, 1997; Soto *et al.*, 2008). These studies failed to show the predicted increase in FA, illustrating the inconsistency between studies using FA as a stress indicator.

However, it is worth discussing, in the light of our results, the fact that the alkaloid concentration close to the native concentration in cactus tissues did not induce significant reductions in fitness estimators, including FA. In fact, the lowest concentration of the alkaloid fraction produced a slight positive impact on adult size and induced a very low incidence of venation anomalies, suggesting a transition between toxicity and adaptation. In contrast, greater doses of alkaloids produced significant responses in terms of fitness reduction. This biphasic dose–response, with positive slopes below and negative slopes above the threshold dose (break point), suggests a hormetic response. Hormesis refers to situations in which a stressor produces stimulating effects at doses below the toxicity threshold and causes toxic/inhibition effects at doses above the toxicity threshold (Calabrese *et al.*, 1999; Calabrese & Baldwin, 2003). Mechanisms proposed to account for hormesis were defined in Timbrel (2009) as: 'Low doses of stress stimulate repairing or protective mechanisms which would be followed by overcompensation. Hence, there is a reduced level of pathological change. As the dose increases, the damage and dysfunction are less easily repaired or there is less reserve capacity until the processes are overwhelmed, setting a threshold for toxicity'. This nonlinear dose/response has been described across a wide range of taxa exposed to a diverse array of chemical and environmental stressors (Forbes, 2000). Actually, many mechanisms that account for hormesis, such as the induced expression of CYP enzymes encoded by genes of the P450 family (Timbrel, 2009), have been shown to be involved in the response to exposure to cactus alkaloids in cactophilic *Drosophila* living in the Sonora desert (Fogleman & Danielson, 2001; Matzkin, 2012). For instance, CYP6D2 is involved in the metabolism of phenethylamine alkaloids analogs to mescaline (Rendic, 2002). Likewise, misexpression of CYP2D6-T107A in *D. melanogaster* has been shown to result in wing patterning defects (Reiter *et al.*, 2000). Thus, implication of CYP enzymes in the responses to alkaloids in South American cactophilic *Drosophila* deserves meticulous evaluation.

Our results partially agree with the hypothesized interaction between stress, fitness and FA in a population of susceptible and resistant individuals, proposed by Floate & Fox (2000). These authors remarked that under low stress, increasing FA levels of susceptible individuals raises the average FA level, whereas under moderate stress, average FA declines as susceptible individuals are removed, leaving only robust individuals. Any further increase in stress has no effect on FA because of the differential mortality effect of susceptible genotypes.

In our case, the activation of detoxification mechanisms, if shaped by adaptive evolution, may enable individuals to buffer the stress impact maintaining relatively low levels of FA, with a fitness increment. Under moderate stress, susceptible individuals would reach the detoxification threshold, increasing average FA, and a concomitant decrease in mean fitness. Under high stress, only robust genotypes would survive exhibiting the same FA level as individuals under low stress, but with low viability.

Nevertheless, in the case of shape, fluctuating asymmetry showed an increasing tendency with the alkaloids' dose beyond the lowest concentration, the results did not reveal a consistent pattern with size FA. Our findings are in line with the proposal that the genetic bases of wing shape and size are relatively independent (Carreira *et al.*, 2011) and point to the need of a meticulous analysis of its impact on fitness and developmental processes.

Although, these differences could be due to the dose range used, together with a differential sensitivity for size and shape (Breuker *et al.*, 2006), further experiments should use a wider range of concentrations of the potential stressor to avoid hormesis as another confounding factor in the use of FA as environmental monitor.

The morphological anomalies found in the wings of treated flies may be considered, along with the decreased viability, as an indicator that alkaloids elicited dose-dependent increased levels of stress. Known examples of highly toxic/teratogenic alkaloids include atropine, caffeine, nicotine, belladonna, digitalis, strychnine

(Jackson *et al.*, 2002) and mescaline (Hirsch & Fritz, 1981). The latter is a main constituent of the alkaloid fraction extracted from *T. terschekii*. Our results on the distribution of anomalies among treatments have shown that the frequency of anomalies is dependent on the alkaloids dose, but are not related to sex. In addition, the types of anomalies found did not respond to a quantitative effect of the stressor, suggesting its expression as a result of cryptic genetic differences. This is in line with numerous studies that have documented an increase in phenotypic variation under stress, suggesting the release of cryptic genetic variation normally hidden during ordinary conditions but expressed when canalization systems are challenged (Badyaev, 2005; Dworkin, 2005). Indeed, ectopic wing veins commonly formed in specific genetic backgrounds have been related with atavisms, suggesting that *Drosophila* may retain information for additional veins (Blair, 2007).

In *D. melanogaster*, differentiation of the posterior cross-vein takes place later in wing development and it was found that mutations that eliminate cross-veins do not affect longitudinal veins (Klingenberg & Zaklan, 2000; Blair, 2007). This supports the developmental constraint hypothesis, which states that structures involved in early development will be under stronger purifying selection due to deleterious pleiotropic effects spreading along the ontogeny (Artieri *et al.*, 2009). Thus, inherent constraint imposed by developmental programmes on the phenotype would limit the kinds of possible morphological variation (Guerra *et al.*, 1997). Our observations that longitudinal veins have not been affected (except few cases of anastomosis in LV4; Fig. 2c), along with the discrete nature of the anomalies, are in line with this explanation, letting us to propose a mechanism by which hierarchical selection pressure during ontogeny could lead to more or less variable traits (or modules) depending on the time that the genes are expressed, so variations linked to genes that are expressed much later in development could be reflected in the pattern of the possible phenotypic variants. However, the presented experiment was not designed to investigate these alternatives and a study of gene expression through developmental stages is needed to test this hypothesis.

The relationship between canalization and developmental stability assessed by the levels of variation within and among individuals showed that for size, individual variation did not increase with alkaloid doses, whereas shape individual variation increased in all alkaloid concentrations. This disengaged relationship was further strengthened by the lack of significant correlation between among-individual size variation and its FA (within variation), which contrasts with the significant correlation for shape individual variation and its FA levels. This last finding is consistent with a previous study (Breuker *et al.*, 2006) that holds that the relationship between individual variation and FA is trait specific due to differences in sensitivity during developmental processes. However, the observation of phenodeviant wings, in our work, gave us an unexpected chance to test more directly the relationship between canalization and developmental stability. Supporting the previous observation of a decoupled pattern, the tests of asymmetries between canalized and decanalysed phenotypes showed that although size FA levels were similar in individuals with normal and abnormal wing venation patterns, shape FA significantly increased in the latter group. This is in coincidence with Waddington's idea that canalization and developmental stability are at least partly different processes and functionally distinct (Waddington, 1957; Debat *et al.*, 2001, 2009). If a single mechanism operates, one would expect that individuals with anomalous wing venation patterns expressed greater FA, and vice versa. The mixed pattern observed in our results agrees with other studies, including *Drosophila* wings, that suggests the hypothesis previously stated that canalization and developmental stability are trait specific (Hoffman & Woods, 2003; Debat *et al.*, 2009).

Finally, given the confusion on the exact definition of the concepts and terms describing developmental stability, canalization and phenotypic plasticity, (Debat & David, 2001; Nijhout & Davidowitz, 2003; Dworkin, 2005), a definition of 'robustness' is needed to interpret our results. Although homoeostasis describes the maintenance of a constant physiological state, canalization refers to the ability of developmental processes to achieve a defined endpoint despite perturbations, even through an alternative route (Rutherford, 2000). Under this definition, development stability is not a synonym of homoeostasis or canalization, although they are often used interchangeably (Debat & David, 2001; Nijhout & Davidowitz, 2003). Instead, it is the output of two related aspects through a developmental process, the level of homoeostasis during the developmental programme (related to the genetic and/or environmental stress) and the degree of canalization of the trait (e.g. developmental constraint). Thus, a 'robust' genotype may achieve the definitive phenotype either by high canalization in spite of low homoeostasis or by low canalization but high homoeostasis. This latter scenario may provide a suitable explanation to our observations as phenotypic plasticity may act as an organism's 'buffer' capacity to accommodate the stress-induced variation through alternative developmental routes (favouring developmental homoeostasis) in low canalized traits.

In conclusion, fluctuating asymmetry does not seem to represent an easy and straightforward indicator of fitness, especially because of its low explanatory coefficients, trait specificity and subtle differences along with the many confounding factors such as developmental selection, hormesis and phenotypic plasticity. However, the search for sensitive traits to phenotypic deviation

under stress could be a much better indicator with possible important evolutionary implications.

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Table A1 Principal results of the ANOVAS for the segmented linear regression analyses (with and without break-point) testing for wing size as a function of the concentration of the alkaloid fraction in the rearing medium. For alkaloid concentrations below the break-point, the best functions fitting the data were $y = 0.43 + 17.9x$ for females and $y = 0.01 + 16.6x$ for males while the functions producing the best fit for concentrations greater than the break point were $y = -0.8 - 19.5x$ for females and $y = -0.3 + 16.9x$ for males.

	SSD	d.f.	SD	*F*
Females				
Explained by linear regression	6.50	1	2.54	17.48**
remaining unexplained	34.20	92	0.60	
Extra explained by break-point	9.04	3	1.73	10.66**
remaining unexplained	25.15	89	0.52	
Total explained by break-point	15.54	4	1.96	13.74**
remaining unexplained	25.15	89	0.52	
Males				
Explained by linear regression	2.30	1	1.51	12.11
remaining unexplained	15.00	79	0.43	
Extra explained by break-point	0.62	2	0.55	1.66**
remaining unexplained	14.37	77	0.42	
Total explained by break-point	2.92	3	0.98	5.21**
remaining unexplained	14.37	77	0.42	

$^{**}P < 0.01$.

Table A2 Size cellular basis resume results for treatments and sex. Mean and Standard deviation for Cell area indexes, Cell numbers indexes and wing centroid sizes.

		Cell area ($\times 10^{3}$)		Cell number ($\times 10^{-6}$)		Wing size ($\times 10^{-17}$)	
Treatment	Sex	Mean	SD	Mean	SD	Mean	SD
Control	Females	0.86	0.07	1.55	0.10	1.79	0.05
	Males	0.75	0.04	1.47	0.13	1.66	0.04
A1	Females	0.87	0.06	1.55	0.15	1.83	0.04
	Males	0.76	0.08	1.50	0.11	1.67	0.05
A2	Females	0.85	0.05	1.51	0.12	1.79	0.06
	Males	0.76	0.06	1.50	0.10	1.62	0.03
A3	Females	0.82	0.09	1.60	0.15	1.71	0.05
	Males	0.72	0.07	1.49	0.18	1.61	0.04

Table A3 Resume results for Size and FA indexes. Mean and Standard deviation for size were multiplied by 10^{-17} while mean and FA1 standard deviation were multiplied by 10^{-15}.

Treatment	*N*	Mean size	Size SD	Mean FA1	FA1 SD	Mean FA2	FA2 SD	Size FA10	Shape FA10
Control	44	1.720	0.080	1.390	0.953	0.008	0.005	1.480	0.954
A1	52	1.753	0.097	1.667	1.407	0.009	0.006	1.579	0.940
A2	55	1.709	0.104	2.098	1.395	0.012	0.008	1.933	1.332
A3	24	1.687	0.060	1.491	1.197	0.008	0.007	1.337	1.313

Table A4 Principal results of the ANOVA for the segmented linear regression analysis (with and without break-point) testing for the relationship between FA (FA1 index) as a function of the concentration of the alkaloid fraction in the rearing medium. For alkaloid concentrations below the break-point, the best function fitting the data was $y = 1.22 + 0.58x$ ($P < 0.001$, $N = 96$), while the function producing the best fit for concentrations greater than the break point was $y = 3.92 - 1.21x$ ($P < 0.001$, $N = 79$).

	SSD	d.f.	SD	*F*
Explained by linear regression	500	1	22.36	3.41
remaining unexplained	25 300	173	12.09	
Extra explained by break-point	668	3	14.92	1.53**
remaining unexplained	24 631	170	12.03	
Total explained by break-point	1168	4	17.08	2.01**
remaining unexplained	24 631	170	12.03	

**$P < 0.01$.

Table A5 Principal Component Analysis (individuals Procrustes): First 7 PCs.

	Eigenvalues	% Variance	Cumulative %
Control	0.00005554	26.313	26.313
	0.00004775	22.623	48.936
	0.00003892	18.44	67.376
	0.00002383	11.288	78.664
	0.00001879	8.9	87.563
	0.00001388	6.576	94.139
	0.00000562	2.665	96.804
A1	0.00009381	36.122	36.122
	0.00005463	21.036	57.158
	0.00004158	16.011	73.169
	0.00002889	11.123	84.292
	0.00001369	5.273	89.565
	0.00001275	4.909	94.474
	0.00000523	2.013	96.487
A2	0.00009031	30.61	30.61
	0.0000701	23.761	54.37
	0.00006064	20.556	74.927
	0.00002558	8.67	83.596
	0.00002235	7.576	91.172
	0.00001184	4.014	95.186
	0.00000519	1.76	96.946
A3	0.00009072	34.988	34.988
	0.00006466	24.937	59.924
	0.00004757	18.347	78.272
	0.00002217	8.551	86.823
	0.00001657	6.39	93.212
	0.00000663	2.558	95.77
	0.00000437	1.684	97.455

Table A6 Canonical Variate Analysis. (a) Classification criterion: treatment. Sex (F: Females; M: Males) (b) Variation among groups. scaled by the inverse of the within-group variation (c) Procrustes distances among groups (d) *P*-values from permutation tests (10 000 random permutation rounds) for Procrustes distances among groups.

Groups	Observations	
(a)		
1.	A1.F	27
2.	A1.M	25
3.	A2.F	29
4.	A2.M	26

Table A6 (*Continued*)

Groups	Observations	
5.	A3.F	17
6.	A3.M	7
7.	CONTROL.F	21
8.	CONTROL.M	23

	Eigenvalues	% Variance	Cumulative %
(b)			
1.	5.4531136	80.84	80.84
2.	0.71346925	10.577	91.417
3.	0.21757649	3.225	94.642
4.	0.15593493	2.312	96.954
5.	0.0967668	1.435	98.389
6.	0.06179191	0.916	99.305
7.	0.04690365	0.695	100

	A1. F	A1. M	A2. F	A2. M	A3. F	A3. M	CONTROL. F
(c)							
A1. M	0.013						
A2. F	0.011	0.007					
A2. M	0.023	0.011	0.014				
A3. F	0.021	0.011	0.011	0.007			
A3. M	0.034	0.022	0.024	0.013	0.014		
CONTROL. F	0.015	0.027	0.025	0.036	0.035	0.047	
CONTROL. M	0.007	0.018	0.016	0.027	0.026	0.038	0.0109

	A1. F	A1. M	A2. F	A2. M	A3. F	A3. M	CONTROL. F
(d)							
A1. M	< 0.0001						
A2. F	0.0004	0.1880					
A2. M	< 0.0001	0.0118	< 0.0001				
A3. F	< 0.0001	0.0191	0.0143	0.2937			
A3. M	< 0.0001	0.0001	0.0002	0.0737	0.039		
CONTROL. F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
CONTROL. M	0.0347	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0003