

# Experimental hybridization in allopatric species of the *Drosophila repleta* group (Diptera: Drosophilidae): implications for the mode of speciation

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The Pleistocene refugia theory proposes that recurrent expansions and contractions of xerophytic vegetation over periods of climate change affected the evolution of cactophilic *Drosophila* in South America. The resulting demographic fluctuations linked to the available patches of vegetation should have been prone to bottlenecks and founder events, affecting the fate of gene pool dynamics. However, these events also promoted the diversification of cacti, creating an ecological opportunity for host specialization. We tested the hypothesis of ecological speciation in the *Drosophila buzzatii* group. We assessed adaptive footprints and examined the genetic architecture of fitness-related traits in the sibling allopatric species *D. koepferae* and *D. antonietae*. The results are in line with the idea that these species evolved under different ecological scenarios. Joint-scaling analysis comparing both species and their hybrids revealed that additive genetic variance was the major contributor to phenotypic divergence, but dominance, epistasis and maternal effects were also important factors. Correlation analysis among functionally related traits suggested divergent selection on phenotypic integration associated with fitness. These findings support the hypothesis of adaptive evolution driving the phylogenetic radiation of the group through independent events of host shifts to chemically complex columnar cacti.

ADDITIONAL KEYWORDS: adaptation – ecological specialization – experimental design – genetic architecture – introgression – phenotypic integration.

## INTRODUCTION

Understanding the mode of speciation in closely related species is difficult, often hampered by a lack of knowledge of the past and present ecological conditions as well as insufficient description of the resulting genetic consequences (Schluter, 2001). In this regard, the study system of desert *Drosophila* provides a suitable model for speciation research. The distribution ranges of the species are well known, the specificity of host plants used in nature has been well documented, genetic, physiological and morphological peculiarities have been described in detail, and the

phylogenetic relationships have been largely resolved (e.g. Barker & Starmer, 1982; Fogleman & Danielson, 2001; Richmond, Johnson & Markow, 2012). An additional advantage is that these species are at different stages of divergence and can hybridize in the laboratory, providing a powerful experimental tool to assess the strength of selection in segregating traits (e.g. I. M. Soto *et al.*, 2007; Bono & Markow, 2009; Jennings & Etges, 2010). This approach provides a source of genetic diversity, increasing the variance of fitness, while allowing the dissection of environmental and genetic effects in multiple traits independently (see Lexer, Randell & Rieseberg, 2003).

Most radiations of this group were related to the use of cacti as breeding sites (especially the prickly pears), after the central region of South America became warm

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and dry due to uplift of the Andes 17 Mya (Oliveira *et al.*, 2012). During this process, the *Drosophila buzzatii* cluster (*D. repleta* group) originated, giving rise to a group of seven endemic species. Their obligate ecological association with cacti implies an evolutionary history coupled with the diversification of the Cactaceae, promoted by cycles of expansion and retraction of dry areas during Quaternary glacial periods (Manfrin & Sene, 2006). Yet, whether divergence between these closely related species is the outcome of ecological specialization or a mere reflection of evolution by stochastic events (i.e. drift/mutation) remains contentious. On the basis of observed diversification patterns, allelic frequencies, chromosomal rearrangements and morphological traits, previous authors have suggested that the recent diversification of the *D. buzzatii* cluster was due to vicariant events driven by geographical barriers (Morales-Hojas & Vieira, 2012; Franco & Manfrin, 2013). However, recent analysis of patterns of host plant utilization showed that the use of *Opuntia* is common throughout the phylogeny and that host shifts to chemically complex columnar cacti occurred several times independently (Oliveira *et al.*, 2012). This line of evidence, along with the observation of evolved genetic tradeoffs for host-plant utilization, supports the hypothesis of ecological specialization as a major force driving phenotypic divergence (Fernandez-Iriarte & Hasson, 2000).

We tested the hypothesis of ecological speciation in the *D. buzzatii* cluster by examining the ecological components of fitness in two allopatric species (and their hybrids) inhabiting contrasting environments. *Drosophila koepferae* Fontdevila *et al.*, 1988 uses columnar cacti of the genus *Trichocereus* in the arid Andean highlands as its primary host, while *D. antonietae* Tidon-Sklorz & Sene 2001 breeds in columnar cacti of the genus *Cereus* in the Atlantic forests. Both species, however, retain the use of *Opuntia* spp. as secondary hosts (Manfrin & Sene, 2006). Although their phylogenetic relationship has not been fully resolved, estimated divergence time within the *D. buzzatii* cluster nodes ranges from 0.7 to 4.6 My (Oliveira *et al.*, 2012). Depending upon the trait under study, *D. koepferae* has been included in an isolated lineage with *D. buzzatii* or in the so-called *D. serido* subcluster with *D. antonietae* (Manfrin & Sene, 2006). The long-standing phylogenetic discordance of the *D. buzzatii* cluster was first interpreted as the result of past introgression events, and more recently as the action of selection forces and shared ancestral polymorphisms (Franco *et al.*, 2015).

We approached this problem by investigating the phenotypic response of genetic organization under alternative breeding environments. We measured larval viability, development time and wing morphology

in purebreds, hybrids and backcrosses of *D. koepferae* and *D. antonietae* reciprocally raised in each of their host plants. This approach allowed us to assess the strength of divergent natural selection by contrasting the larval performance of each species reared in native versus exotic hosts. Also, and most meaningfully for this study, we used the data from interspecific crosses in analyses of generation means to estimate the main genetic effects underlying the evolution of divergent adaptive traits. We also inspected sex-specific differences to evaluate Haldane's rule in this species pair (Haldane, 1922). Finally, we estimated the level of integration among fitness-related traits as a distinct evolutionary aspect. Some authors have suggested that functional and/or developmental constraints could have stronger effects for phenotypic diversification than environmental factors, while others hold that uniform environments may lead to similar patterns of covariance among traits (Pigliucci, 2003; Wagner & Laubichler, 2004). Therefore, the assessment of genetic and environmental components of phenotypic integration underlying overall fitness might be relevant to the study of the mode of speciation.

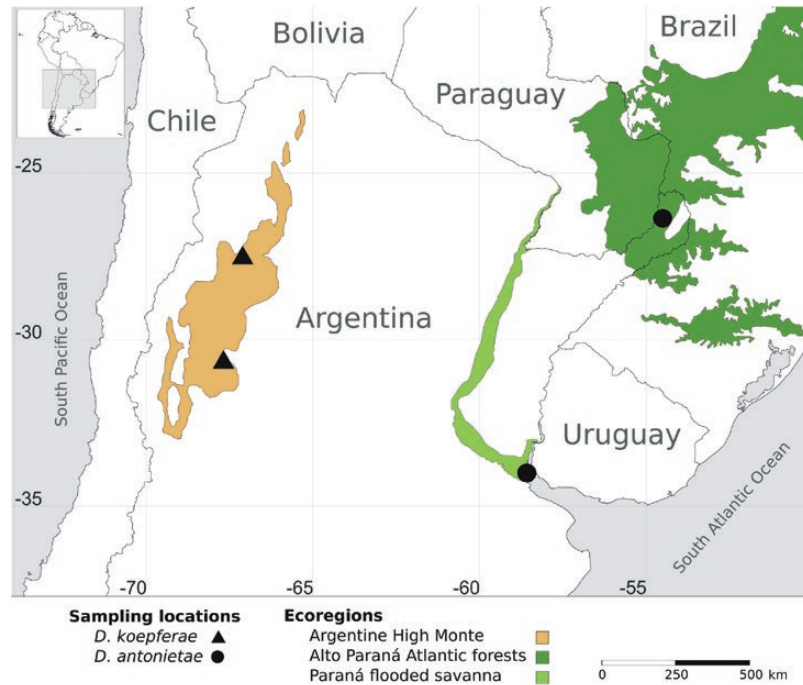
## MATERIAL AND METHODS

### SAMPLE COLLECTION

Natural populations of *Drosophila* were sampled in two locations of each parental species within their ranges of distribution in Argentina (separated by > 1000 km) during the austral summer of 2014–2015. *Drosophila koepferae* was collected in the mountain plateaus of the Monte desert region, whereas *D. antonietae* was sampled in the Atlantic forests and Paraná Delta (Fig. 1). The Paraná Delta consists of a network of islands formed by river sediments, providing the region with Amazonian wildlife (Aceñolaza *et al.*, 2004). Species identification was performed by inspection of male genitalia of the progeny of wild females and maintained in mass cultures with standard medium for five generations prior to experiments. In the Monte region we also collected samples of the main cactus hosts of *D. koepferae* (*O. sulphurea* G. Don and *T. terscheckii* Britton & Rose), while hosts of *D. antonietae* (*O. monacantha* Haworth and *C. hildmaniannus* Schum) were sampled in the area surrounding the Paraná Delta (cacti were stored at  $-20^{\circ}\text{C}$ ).

### EXPERIMENTAL DESIGN

We used a 'reciprocal transplant' design, exposing inter- and intraspecific hybrids to all cactus hosts (Lexer *et al.*, 2003). A semi-natural medium of each cactus was prepared according to De Panis *et al.*



**Figure 1.** Map of South America showing sampling locations of *D. koepferae* and *D. antonietae* with the three main ecoregions covered in their distribution range (Olson *et al.*, 2001).

(2016): fresh pieces of each cactus species were homogenized in a blender and batches of 6 g (supplemented with 0.2 g of 40 g/L of killed yeast extract) were transferred to standard glass vials. We established all possible homospecific and heterospecific crosses between *D. koepferae* (Dk) and *D. antonietae* (Da):  $\sigma\text{Dk} \times \text{♀Dk}$ ,  $\sigma\text{Dk} \times \text{♀Da}$ ,  $\sigma\text{Da} \times \text{♀Da}$  and  $\sigma\text{Da} \times \text{♀Dk}$ . We then backcrossed (BC) the female  $F_1$  hybrids with their homospecific father.  $\text{BC}_{\text{Dk}}: \text{♀} (F_1 \sigma\text{Dk} \times \text{♀Da}) \times \sigma\text{Dk}$ ;  $\text{BC}_{\text{Da}}: \text{♀} (F_1 \sigma\text{Da} \times \text{♀Dk}) \times \sigma\text{Da}$ . To increase genetic diversity and avoid spurious effects (e.g. peculiar genotypes and/or heterosis of inbred crosses) a mass intercrossing design was performed. Each cross was obtained by releasing ~250 mated females (with a similar number of males) in egg-collecting chambers with Petri dishes containing egg-laying medium (2% agar). After 12 h Petri dishes were removed and placed in incubating chambers at 25 °C for a further 12 h until eggs hatched. Batches of 50 first-instar larvae of each cross were then seeded in five culture vials per treatment (replicates) with *O. sulphurea* (*O.s.*), *O. monacantha* (*O.m.*), *T. terscheckii* (*T.t.*) or *C. hildmaniannus* (*C.h.*) media. Vials were incubated at constant temperature with a 12:12-h light/dark photoperiod. Adults were collected daily as they emerged and sexed under light  $\text{CO}_2$  anaesthesia. Hybrids were verified in two different ways. First, we introduced adults of each  $F_1$  hybrid cross into egg-collecting chambers to confirm the absence of progeny due to contamination (i.e.  $F_1$

males are sterile). In addition, we extracted salivary glands from a sub-sample of third-instar larvae that remained in Petri dishes to inspect the typical asynapses in the polytene chromosomes (I. M. Soto *et al.*, 2007; E. M. Soto *et al.*, 2008).

#### TRAITS SCORED

We measured larval viability as a direct indication of adaptation to the host cactus, as this is the most immediate factor affecting early life cycle, and also development time and wing size as these traits are related to adult fitness (e.g. reproductive success, longevity, fertility and stress tolerance) and are accurate predictors of environmental quality in *Drosophila* (Egtes, 1990; I. M. Soto *et al.*, 2014). In addition, we assessed changes in wing shape because this is a taxonomic trait, is related to host shifts and is predicted to be involved in sexual selection and dispersal ability (Moraes *et al.*, 2004; Menezes *et al.*, 2013).

Sex ratio and viability were estimated by counting emerged males and females from each experimental vial. Chi-square tests were used to analyse the effect of cactus (four levels) and interspecific crosses (six levels) on the sex ratio (Wilson & Hardy, 2002). The significant effect of multiple tests was corrected by Bonferroni's correction. Development time was estimated as the time (in hours) elapsed since the time of transfer of first-instar larvae until adult emergence (individuals

as replicates). Larval viability and development time scores were angularly and log-transformed, respectively, prior to statistical tests (Zar, 1996).

Adult morphology was studied by removing the right wings of female adults of each combination of cross by cactus (total of 642 wings). Wings were mounted on slides with dibutyl phthalate in xylene (DPX, Sigma Aldrich) and photographed in a compound microscope at 20× magnification (Nikon E200). After image digitalization, we used geometric morphometric techniques to analyse shape and size separately (Bookstein, 1996). Landmark positioning was performed in TpsDIG2 software (Rohlf, 2015) at the junctions of the most representative veins (details in Padró *et al.*, 2014). Wing size was estimated as the centroid size, defined as the square root of the sum of the squared distances of landmarks to the centroid of the configuration (Dryden & Mardia, 1998). Wing shape variation was determined by the least squares Procrustes superimposition method to generate variance–covariance matrices. To find the shape features that best distinguish the effects among cacti and crosses, we performed a canonical variate analysis (CVA) by testing pairwise comparisons on Procrustes distances among groups (10 000 permutations). Morphometric analyses were carried out in MorphoJ software (Klingenberg, 2011).

Since environmental adaptation may lead to different life histories with equivalent fitnesses, we investigated overall fitness by the relative performance index (RPI) according to the formula (Krebs & Barker, 1993; I. M. Soto *et al.*, 2014):  $RPI = \text{viability} \times \text{wing size} / \text{developmental time}$ . Because RPI combines the effect of multiple traits that may not be independent, we looked at the correlation patterns between traits (i.e. phenotypic integration) in hybrids and their parental species in different host plants (Pigliucci, 2003). We used path analysis, which allows us to decompose the correlation of multiple variables into a sum of direct and indirect effects through the other independent variables (Arnold, 1983; Callahan & Waller, 2000).

#### ANALYSIS OF VARIANCE

Genetic and environmental effects in each fitness trait were evaluated via two-way ANOVAs with ‘Cactus’, ‘Cross’ and the interaction ‘Cactus × Cross’ as fixed factors. This analysis was conducted for larval viability, development time, wing size and RPI. Planned contrasts between parental species were conducted to assess adaptive differences when larvae develop in native/foreign hosts. Where significant differences among species were found, we proceeded to further investigate the genetic basis of trait divergence (see below).

Morphological analysis of shape variation was performed by means of principal component analysis

on the landmark variance–covariance matrices and PC scores were then used as dependent variables in MANCOVAs testing for differences among ‘Cactus’ and ‘Cross’ (centroid size was used as a covariate).

#### GENETIC INFERENCE OF PHENOTYPIC VARIATION

We assessed the genetic bases underlying larval viability, development time, size and overall fitness by joint-scaling tests of phenotypic means, a method consisting of a weighted least squares multiple regression design for scaling populations (purebreds, hybrids and backcrosses) with different variances (Lynch & Walsh, 1998; Carroll *et al.*, 2001). We tested additive, dominance, epistatic and maternal effects for the divergent traits among parental species (i.e. those that were significant in the planned contrasts). Pure additive effects will yield hybrids with average phenotypes between parental species, whereas dominance will cause a deviation toward one parental species. Digenic epistasis (deviation from additivity and dominance) comprised additive × additive [aa], dominance × dominance [dd] and additive × dominance [ad] effects, while the maternal effects (deviation towards the maternal line) reflect cytoplasmic effects. Model parameters are shown in the Supporting Information (Table S1). We tested the additive-dominance model incorporating an extra parameter if it improved the explanatory power of the analysis. To find the model for each trait that best described the data, we performed a full model selection analysis including all parameters. The reduced models were chosen according the lowest Bayesian information criterion.

All statistical analyses were performed using the generalized linear model as implemented in the STATISTICA 6.0 software package (Statsoft, 2001) except for chi-square tests, path analysis and selection model tests, which were performed in Infostat software v2016.

## RESULTS

### SEX RATIO

A main result of the experimental crosses was the observed sterility of all  $F_1$  hybrid males, indicating the evolution of post-mating reproductive isolation among species. In addition, the sex ratio test showed significant differences among crosses ( $\chi^2 = 26.32$ ,  $df = 5$ ,  $P < 0.001$ ), but not among cactus ( $\chi^2 = 6.54$ ,  $df = 3$ ,  $P = 0.08$ ). Multiple analyses of each cross revealed a sex bias in the hybrid cross  $\sigma Dk \times \text{♀} Da$ , with a mean of 55.3% males ( $\chi^2 = 7.27$ ,  $df = 1$ ,  $P < 0.05$ ) and in  $BC_{Dk}$  with 56.5% males ( $\chi^2 = 12.14$ ,  $df = 1$ ,  $P < 0.01$ ) while all other crosses remained fairly equal ( $P > 0.05$ ; Fig. S1).



## FITNESS TRAITS

The two-way ANOVAs performed on fitness-related traits showed significant interaction between crossing type and rearing cactus in all cases (Table S2).

## LARVAL VIABILITY

Planned contrasts among species revealed that *D. koepferae* was more viable than *D. antonietae* when raised in *T. terscheckii* ( $F_{1,39} = 37.22, P < 0.001$ ) while the opposite occurred in *C. hildmannianus* ( $F_{1,39} = 7.05, P = 0.01$ ). Moreover, both *Drosophila* species tended to achieve similar viabilities when reared in either of the prickly pear species (*O.m.*:  $F_{1,39} = 0.52$ ; *O.s.*:  $F_{1,39} = 2.13$ ;  $P > 0.05$  in both cases). Analysis of generation means revealed a hybrid breakdown in the BC<sub>Da</sub> when reared in *T. terscheckii* (0 viability) and in the BC<sub>Dk</sub> when reared in *C. hildmannianus* (mean viability of 0.11; Fig. 2A, B). Nonetheless, most of the variance in the regressions was explained by additivity alone (Table 1, Fig. 3).

## DEVELOPMENTAL TIME

Planned contrasts showed that *D. koepferae* consistently developed faster than *D. antonietae* in all breeding resources (*C.h.*:  $F_{1,928} = 502.74$ ; *O.m.*:  $F_{1,928} = 130.71$ ; *T.t.*:  $F_{1,928} = 44.49$ ; *O.s.*:  $F_{1,928} = 166.47$ ;  $P < 0.001$  in all cases), indicating conspicuous genetic differences among species. In addition, all interspecific hybrids showed noticeable transgressive developmental times (i.e. values outside the range of both parent species), causing deviations from the additive model expectations (Fig. 2C, D). An improved fit to the standard genetic model was achieved by adding maternal effects, although its explanatory contribution was significant only in *T. terscheckii* and *O. monacantha* (Table 1). Additive-dominance effects had fairly similar contributions in *C. hildmannianus*, explaining nearly all of the variance, while in *T. terscheckii* the maternal effect accounted for almost half of the total variance. For the prickly pears, additive effects explained almost the entire variance in *O. monacantha*, whereas dominance was the major contributor in *O. sulphurea* (Fig. 3).

## WING SIZE

*Drosophila koepferae* attained larger wing sizes than *D. antonietae* when raised in *C. hildmannianus* ( $F_{1,206} = 24.43, P < 0.001$ ) whereas the opposite trend was observed when it was raised in *T. terscheckii* ( $F_{1,206} = 6.56, P < 0.05$ ; Fig. 2E, F). However, when larvae were raised in *Opuntia* spp., there were no differences among species (*O.m.*:  $F_{1,206} = 1.35$ ; *O.s.*:  $F_{1,206} = 3.78$ ;  $P > 0.05$  in both cases). The genetic model included the maternal line as an explanatory predictor (Table 1), although additive effects explained almost the entire variance (Fig. 3).

## RELATIVE PERFORMANCE INDEX

Our interspecific planned contrasts revealed a noticeable superior performance of *D. koepferae* when raised in *T. terscheckii* ( $F_{1,39} = 39.43, P < 0.001$ ) and of *D. antonietae* when raised in *C. hildmannianus* ( $F_{1,39} = 5.23, P = 0.03$ ). However, no differences among species were found when raised in *Opuntia* hosts (*O.m.*:  $F_{1,39} = 0.64$ ; *O.s.*:  $F_{1,39} = 3.36$ ;  $P > 0.05$  in both cases). The explanatory power of the maternal effect improved the additive-dominance model, especially in *T. terscheckii* (Table 1). Nonetheless, additivity effects explained most of the variance in both species of columnar cacti (Fig. 3).

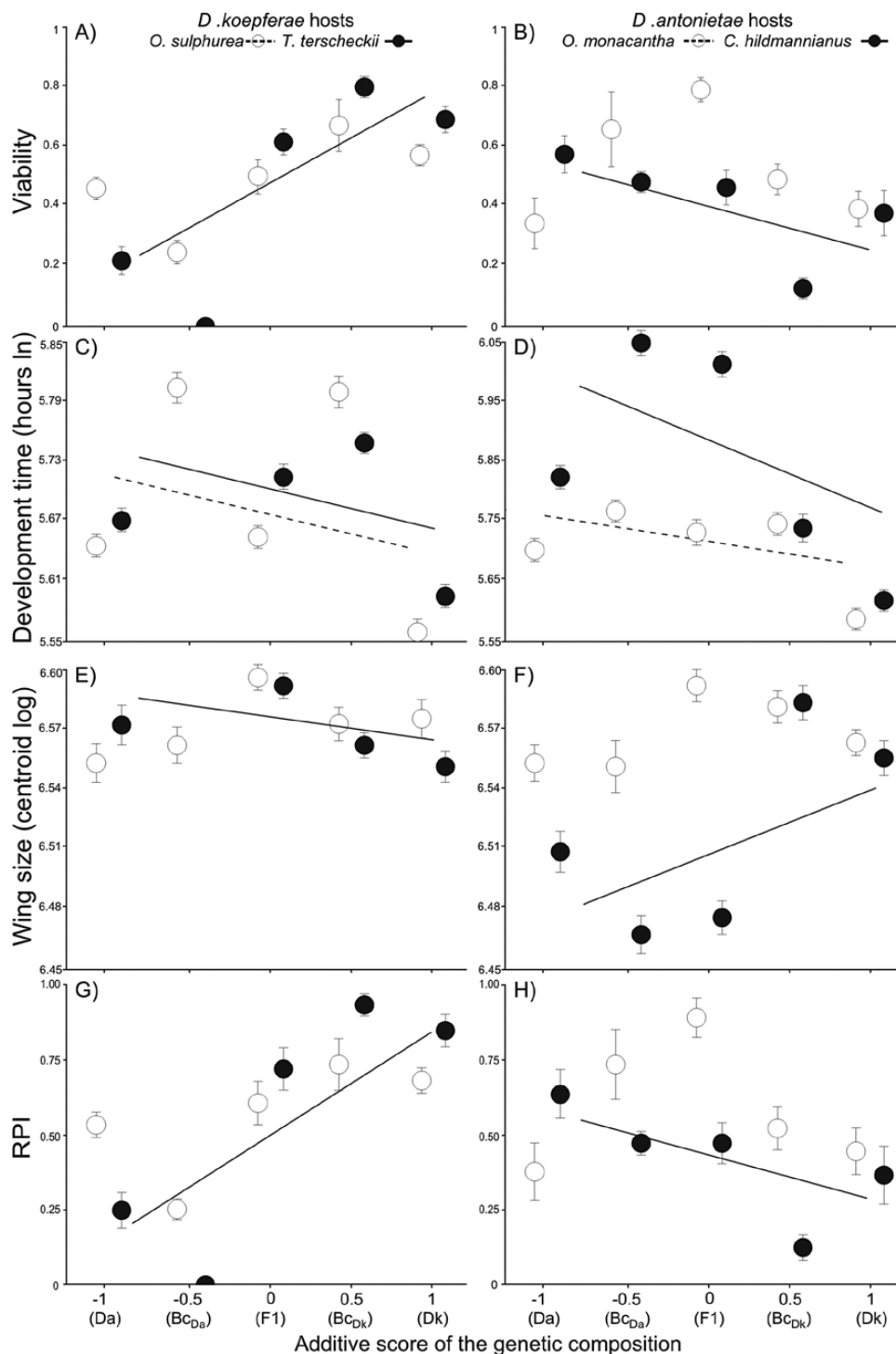
Path analysis revealed that RPI was largely determined by the direct effects of viability ( $r > 0.96, P < 0.001$  in all crosses; Fig. S2). However, in *D. antonietae* development time (partial  $r = 0.50, P < 0.05$ ) and wing size (partial  $r = -0.56, P < 0.05$ ) were also strong contributors to fitness (details in Fig. S2). RPI in  $\sigma$ Dk  $\times$   $\varphi$ Da was also closely related to the effects of wing size (partial  $r = 0.57, P < 0.01$ ) and development time (partial  $r = -0.54, P < 0.01$ ). Correlation between development time and viability was substantial in BC<sub>Dk</sub> as well (partial  $r = 0.60, P < 0.01$ ). Analysis of the effect of cactus revealed that regardless of the species, fitness was also predominantly determined by viability (direct  $r = 0.99, P < 0.001$  in all cases). However, in *O. monacantha* RPI was strongly associated with the indirect effects of development time (partial  $r = 0.44, P = 0.02$ ).

## WING SHAPE

MANCOVAs performed on PC scores detected significant effects of Cross and Cactus (Table 2). In addition, an allometric effect was detected as a shape and size covariate. CVA applied to the Cross effect showed a clear separation among parental species, with the greatest variation in the distal-posterior region (Fig. 4A). Hybrids and backcrosses displayed intermediate values for the first axis (CV1), with F<sub>1</sub> individuals resembling the shape of *D. koepferae*. For the second axis (CV2), F<sub>1</sub> individuals approached the mean shape of *D. antonietae*, whereas backcrosses attained transgressive phenotypes. For the effect of Cactus, the analysis revealed that wing shape was similar when flies bred in *Opuntia* species, but divergent among columnar cacti (Fig. 4B).

## DISCUSSION

Our results showed that *D. koepferae* is ecologically and genetically divergent from *D. antonietae*, supporting the idea that they belong to different lineages within the *D. buzzatii* cluster (Manfrin, De Brito & Sene, 2001;



**Figure 2.** Mean viability (A, B), development time (C, D), wing size (E, F) and relative performance index (RPI) (G, H) as a function of the gene dosage derived from each parental species. Linear trends correspond to the additive model expectation for the primary (solid lines) and secondary hosts (dashed lines) of each parental species (estimated when species significantly differ in their phenotypic means). Standard errors are shown.

Franco *et al.*, 2010). The observation of male sterility in the  $F_1$  offspring is consistent with Haldane's rule and agrees with patterns of rapid evolution found in

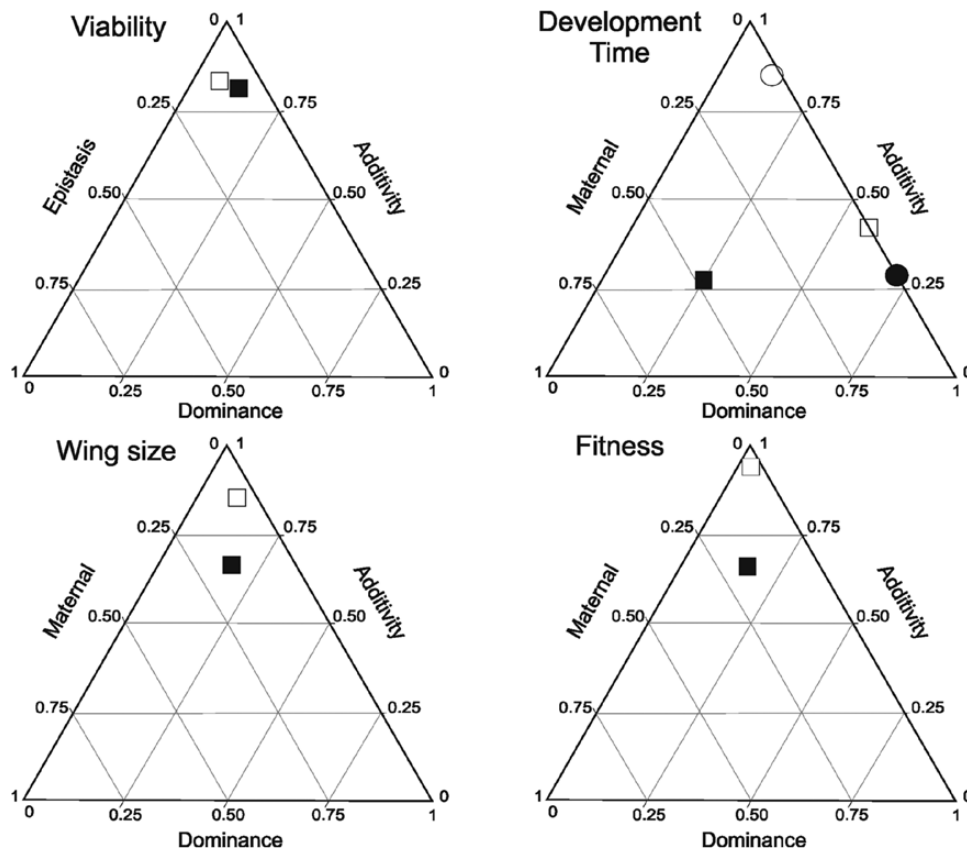
incipient species of *Drosophila*, with hybrid sterility evolving faster than inviability (Wu & Davis, 1993). Nonetheless, the sex-biased survivorship in hybrids

**Table 1.** Summary of weighted least squares multiple regressions ( $F$ -values), estimating genetic effects of phenotypic divergence among allopatric species of *Drosophila*

Trait	Effect/Cactus	<i>T.t</i>	<i>O.s</i>	<i>C.h</i>	<i>O.m</i>
Viability	Additive	440.49***	NS	206.07***	NS
	Dominance	64.51***	NS	15.70***	NS
	Epistasis [dd]	34.36***	NS	24.41***	NS
Development time	Additive	4.59*	272.02***	3239.18***	795.38***
	Dominance	3.93*	655.12***	4275.45***	122.71***
	Maternal	7.87**	0.01	0.01	22.57***
Wing size	Additive	56.19***	NS	261.58***	NS
	Dominance	16.57***	NS	33.62***	NS
	Maternal	13.34***	NS	17.21***	NS
RPI	Additive	79.84***	NS	91.43***	NS
	Dominance	22.00***	NS	1.78	NS
	Maternal	21.89***	NS	1.50	NS

NS, no significant differences between the mean values of parental species (planned contrasts). *T.t*, *T. terscheckii*; *O.s*, *O. sulphurea*; *C.h*, *C. hildmaniannus*; *O.m*, *O. monacantha*. dd, dominance  $\times$  dominance.

\* $P < 0.01$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .


**Figure 3.** Ternary plots showing the proportional contribution of each genetic parameter to the total variance (estimated from joint-scaling analysis) when larvae are exposed to *T. terscheckii* (■), *C. hildmannianus* (□), *O. sulphurea* (●) and *O. monacantha* (○).

from the same mother line agrees with common exceptions to Haldane's rule (Wu & Davis, 1993). Maternal effects in hybrids have been related to the disruption

of co-adapted nuclear–mitochondrial gene complexes, pathogens, symbionts, transposons and non-coding RNAs (Thomas-Orillard, 1984; Labrador *et al.*, 1999;

**Table 2.** MANCOVA results for PC scores of wing shape, testing for Cross and Cactus effects (centroid size as covariate)

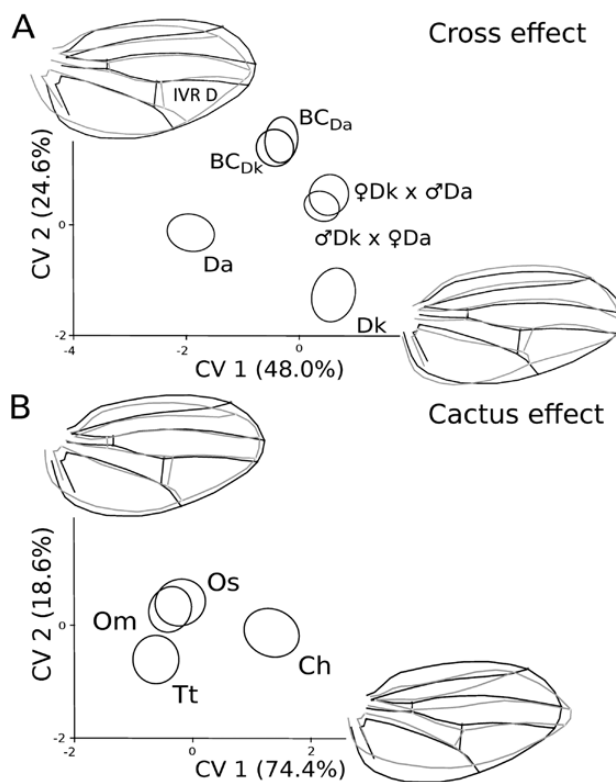
Effect	Wilks	df (numerator)	d.f (denominator)	F
Size	0.68	16	617	18.01***
Cross	0.26	80	2975	12.05***
Cactus	0.67	48	1836	5.56***

\*\*\* $P < 0.001$ .

Sackton, Haney & Rand, 2003; Glaser & Meola, 2010; Chambeyron & Seitz, 2014).

In terms of larval performance, we demonstrated that not all species of cacti represent similar ecological scenarios for these closely related *Drosophila* Species. Rearing in columnar cacti proved to be a stressful environment for non-resident species (Table 1). Moreover, the differential effects of columnar cacti on wing morphology reinforced the idea that these resources exert divergent selection pressures, while prickly pears appear to represent equivalent environments for developing larvae (Table 1; Fig. 4B). This finding is not surprising because according to phylogenetic studies performed in the *repleta* group, the use of *Opuntia* represents the ancestral ecological scenario, whereas the use of columnar cacti could be considered as the apomorphic condition (Oliveira *et al.*, 2012).

A possible explanation for the different abilities of species to use alternative host plants might relate the chemistry of the cactus. For instance, secondary metabolites have been implicated in patterns of adaptive evolution, suggesting that defensive cactus chemicals act as major evolutionary drivers of cactophilic *Drosophila* (e.g. Fogleman & Danielson, 2001; I. M. Soto *et al.*, 2014; De Panis *et al.*, 2016). In North America, allopatric populations of *D. mojavensis* are associated with chemically distinct cacti hosts, demonstrating footprints of positive selection in genes of detoxification metabolism (Matzkin, 2014). *Drosophila mettleri* is another example of genetic differentiation among host races due to selection of alkaloid detoxification mechanisms (Bono *et al.*, 2008). In general, the genus *Opuntia* is rich in phenolics, but has low levels of alkaloids, mostly represented by phenethylamines (Meyer, Mohamed & McLaughlin, 1980; Ma *et al.*, 1986; Stintzing & Carle, 2005). This is the case for *O. monacantha* (Valente *et al.*, 2007), but not for *O. sulphurea* whose chemical profile is similar to proline alkaloids (De Panis *et al.*, 2016). As with many other columnar cacti, *T. terscheckii* is highly rich in phenethylamine alkaloids (Reti & Catrillon, 1951; Corio *et al.*, 2013), although *C. hildmannianus* still lacks a comprehensive chemical analysis.



**Figure 4.** First two canonical variate axes showing mean wing shape differences among crosses (A) and cactus (B). Wing outlines denote shape changes across the axes: black outlines denote extreme negative values, whereas grey outlines denote positive values. The 95% confidence ellipses for each mean are depicted. IVR D, distal-posterior intervein region.

Evidence of high larval lethality in the backcrosses reared in the non-native columnar cactus of the reintroduced species (Fig. 2A, B) strongly suggested the disruption of co-adapted gene complexes, regulation mechanisms and/or favourable epistatic relationships (Lynch & Walsh, 1998; Renaut & Bernatchez, 2011). This pattern is consistent with the idea that the physiological consequences of hybrid breakdown may only be revealed in stressful environments (Hatfield & Schluter, 1999). Moreover, the higher viabilities of the purebreds observed when raised in their primary host suggests that co-adapted genetic elements as well as a particular epigenetic background may provide specific advantages only under certain ecological conditions. In desert species of *Drosophila*, specialization to columnar cacti probably required the integrated action of several genomic elements related to developmental stability in chemically challenging environments (Clarke & McKenzie, 1987; I. M. Soto *et al.*, 2008; Carreira *et al.*, 2008). In this sense, it is interesting to note that *D. koepferae* and *D. antonietae* can be distinguished



by unique chromosomal inversions (Manfrin & Sene, 2006). Notwithstanding this, our study was also coincident with the classical view of additive variance as the main substrate of natural selection (Fisher, 1930), suggesting a complex genetic basis of host specialization. Interestingly, diversifying selection has been predicted to be more effective when adaptive traits are affected by a mix of large- and small-effect alleles (Débarre, Yeaman & Guillaume, 2015). However, our analysis showed no indication of genetic differentiation between *Drosophila* species for the use of alternative *Opuntia* substrates, suggesting the maintenance of shared ancestral polymorphisms (Franco *et al.*, 2015) and/or epigenetic structures (Chambeyron & Seitz, 2014) related to their plesiomorphic rearing condition (Oliveira *et al.*, 2012).

Developmental time appeared to have evolved independently in each parental species (Fig. 2C, D), probably due to the dissimilar range of natural habitat conditions where these species are found: *D. koepferae* inhabits arid environments, where a shorter developmental time should be an adaptive trait, reducing the risk of larval mortality due to desiccation. The observed elevated levels of non-additive variance (Fig. 3) were probably the result of transgressive segregation due to the complementary action of divergent genetic components (Rieseberg, Archer & Wayne, 1999). Transgressive phenotypes were also found in wing morphology (Fig. 4A), a phenomenon that is typical in hybrids and has been considered instrumental in the generation of adaptive radiations (Rieseberg *et al.*, 1999; Lexer *et al.*, 2003). Previous reports speculated that the distal-posterior region (IVR D), involved in wing shape divergence between *D. koepferae* and *D. antonietae* (Fig. 4A), represents independent evolutionary units associated with host-plant adaptation (E. M. Soto *et al.*, 2008; I. M. Soto *et al.*, 2008; Padró *et al.*, 2014). Moreover, the contribution of maternal effects to phenotypic traits has been related to chemical tolerance (Kerver & Rotman, 1987) and epigenetic control of non-coding RNA in *Drosophila melanogaster* (Chambeyron & Seitz, 2014).

The overall performance results suggest that viability selection is a crucial component of fitness in nature. However, it is important to note that host plants may have further implications on other interacting forces not addressed here, such as the evolution of host preference, sexual selection and reproductive isolation (Honda *et al.*, 2016; Muller *et al.*, 2016; E. M. Soto *et al.*, 2017). In fact, phenotypic integration found in *D. antonietae* indicates that selection is also operating on other life stages (Fig. S2). The lack of integration found in *D. koepferae* (Fig. S2) could be understood in terms of either genetic drift or the extreme environment of its columnar host, causing larval survivorship to be the major explanatory factor of reproductive

fitness (I. M. Soto *et al.*, 2014). Interestingly, the environmental effect of increased phenotypic integration when larvae were reared in the main host plant of *D. antonietae* (Fig. S2) suggests a coincident pattern with a history of divergent selection acting on correlation patterns. Overall, these results support the idea that the evolution of trait correlations has a major role in phenotypic divergence.

## CONCLUSION

The greater fitness achieved in each parental species when reared in their preferred columnar cactus is consistent with ecological specialization. The observation of hybrid breakdown and substantial additive variation suggests a complex genetic basis underlying adaptive divergence. Although we found epistatic effects contributing to interspecific divergence in larval survival, we did not find a general pattern of hybrid depression as should be expected by locus incompatibilities under the mutation-order model, supporting the hypothesis of ecological speciation. Thus, although we cannot rule out the possibility that genetic differentiation may have evolved after random genetic drift due to colonization and/or bottleneck events, we can conclude that host plant use was probably a major contributor to phenotypic divergence among these species.

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## REFERENCES

- Aceñolaza PG, Povedano HE, Manzano AS, de Dios Muñoz J, Areta JI, Virgolini ALR. 2004. Biodiversidad del Parque Nacional Pre-Delta. *Miscelánea* **12**: 170.
- Arnold SJ. 1983. Morphology, performance and fitness. *American Zoologist* **23**: 347–361.
- Barker JSF, Starmer WT, eds. 1982. *Ecological genetics and evolution: the cactus-yeast-Drosophila model system*. New York: Academic Press.

- Bono JM, Markow TA. 2009.** Post-zygotic isolation in cactophilic *Drosophila*: larval viability and adult life-history traits of *D. mojavensis*/*D. arizonae* hybrids. *Journal of Evolutionary Biology* **22**: 1387–1395.
- Bono JM, Matzkin LM, Castrezana S, Markow TA. 2008.** Molecular evolution and population genetics of two *Drosophila mettleri* cytochrome P450 genes involved in host plant utilization. *Molecular Ecology* **17**: 3211–3221.
- Bookstein FL. 1996.** Biometrics, biomathematics and the morphometric synthesis. *Bulletin of Mathematical Biology* **58**: 313–365.
- Callahan HS, Waller DM. 2000.** Phenotypic integration and the plasticity of integration in an amphicarpic annual. *International Journal of Plant Sciences* **161**: 89–98.
- Carreira VP, Soto IM, Fanara JJ, Hasson E. 2008.** A study of wing morphology and fluctuating asymmetry in interspecific hybrids between *Drosophila buzzatii* and *D. koepferae*. *Genetica* **133**: 1–11.
- Carroll SP, Dingle H, Famula TR, Fox CW. 2001.** Genetic architecture of adaptive differentiation in evolving host races of the soapberry bug, *Jadera haematoloma*. *Genetica* **112**: 257–272.
- Chambeyron S, Seitz H. 2014.** Insect small non-coding RNA involved in epigenetic regulations. *Current Opinion in Insect Science* **1**: 1–9.
- Clarke GM, Mckenzie JA. 1987.** Developmental stability of insecticide resistant phenotypes in blowfly; a result of canalizing natural selection. *Nature* **325**: 345–346.
- Corio C, Soto IM, Carreira V, Padró J, Betti MI, Hasson E. 2013.** An alkaloid fraction extracted from the cactus *Trichocereus terscheckii* affects fitness in the cactophilic fly *Drosophila buzzatii* (Diptera: Drosophilidae). *Biological Journal of the Linnean Society* **109**: 342–353.
- Débarre F, Yeaman S, Guillaume F. 2015.** Evolution of quantitative traits under a migration-selection balance: when does skew matter? *The American Naturalist* **186**: S37–S47.
- De Panis DN, Padró J, Furió-Tarí P, Tarazona S, Milla Carmona PS, Soto IM, Dopazo H, Conesa A, Hasson E. 2016.** Transcriptome modulation during host shift is driven by secondary metabolites in desert *Drosophila*. *Molecular Ecology* **25**: 4534–4550.
- Dryden IL, Mardia KV. 1998.** *Statistical shape analysis*. London: Wiley.
- Etges WJ. 1990.** Direction of life history evolution in *Drosophila mojavensis*. In: Barker JSF, Starmer WT, MacIntyre RJ, eds. *Ecological and evolutionary genetics of Drosophila*. New York: Springer, 37–52.
- Fernández-Iriarte P, Hasson E. 2000.** The role of the use of different host plants in the maintenance of the inversion polymorphism in the cactophilic *Drosophila buzzatii*. *Evolution* **54**: 1295–1302.
- Fisher RA. 1930.** *The genetical theory of natural selection*. Oxford: Clarendon Press.
- Fogleman JC, Danielson PB. 2001.** Chemical interactions in the cactus–microorganism–*Drosophila* model system of the Sonoran desert. *American Zoologist* **41**: 877–889.
- Fontdevila A, Pla C, Hasson E, Wasserman M, Sanchez A, Naveira H, Ruiz A. 1988.** *Drosophila koepferae*: a new member of the *Drosophila serido* (Diptera: Drosophilidae) superspecies taxon. *Annals of the Entomological Society of America* **81**: 380–385.
- Franco FF, Lavagnini TC, Sene FM, Manfrin MH. 2015.** Mito-nuclear discordance with evidence of shared ancestral polymorphism and selection in cactophilic species of *Drosophila*. *Biological Journal of the Linnean Society* **116**: 197–210.
- Franco FF, Manfrin MH. 2013.** Recent demographic history of cactophilic *Drosophila* species can be related to Quaternary palaeoclimatic changes in South America. *Journal of Biogeography* **40**: 142–154.
- Franco FF, Silva-Bernardi ECC, Sene FM, Hasson ER, Manfrin MH. 2010.** Intra- and interspecific divergence in the nuclear sequences of the clock gene period in species of the *Drosophila buzzatii* cluster. *Journal of Zoological Systematics and Evolutionary Research* **48**: 322–331.
- Glaser RL, Meola MA. 2010.** The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS One* **5**: e11977.
- Haldane JB. 1922.** Sex ratio and unisexual sterility in hybrid animals. *Journal of Genetics* **12**: 101–109.
- Hatfield T, Schluter D. 1999.** Ecological speciation in sticklebacks: environment-dependent hybrid fitness. *Evolution* **53**: 866–873.
- Honda K, Honda Y, Matsumoto J, Tsuruta Y, Yagi W, Ômura H, Honda H. 2016.** Production and sex-pheromonal activity of alkaloid-derived androconial compounds in the danaine butterfly, *Parantica sita* (Lepidoptera: Nymphalidae: Danainae). *Biological Journal of the Linnean Society* **119**: 1036–1059.
- Jennings JH, Etges WJ. 2010.** Species hybrids in the laboratory but not in nature: a reanalysis of premating isolation between *Drosophila arizonae* and *D. mojavensis*. *Evolution* **64**: 587–598.
- Kerver WJM, Rotman G. 1987.** Development of ethanol tolerance in relation to the alcohol dehydrogenase locus in *Drosophila melanogaster*. II. The influence of phenotypic adaptation and maternal effect on alcohol supplemented media. *Heredity* **58**: 239–248.
- Klingenberg CP. 2011.** MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* **11**: 353–357.
- Krebs RA, Barker JSF. 1993.** Coexistence of ecologically similar colonising species. II. Populations differentiation in *Drosophila aldrichi* and *D. buzzatii* for competitive effects and responses at different temperatures and allozyme variation in *D. aldrichi*. *Journal of Evolutionary Biology* **6**: 281–298.
- Labrador M, Farré M, Utzet F, Fontdevila A. 1999.** Interspecific hybridization increases transposition rates of *Osvado*. *Molecular Biology and Evolution* **16**: 931–937.
- Lexer C, Randell RA, Rieseberg LH. 2003.** Experimental hybridization as a tool for studying selection in the wild. *Ecology* **84**: 1688–1699.
- Lynch M, Walsh B. 1998.** *Genetics and analysis of quantitative traits*. Sunderland: Sinauer Associates.

- Ma WW, Jiang XY, Cooks RG, McLaughlin JL, Gibson AC, Zeylemaker F, Ostolaza CN. 1986. Cactus alkaloids, LXI. Identification of mescaline and related compounds in eight additional species using TLC and MS/MS. *Journal of Natural Products* **49**:735–737.
- Manfrin MH, De Brito ROA, Sene FM. 2001. Systematics and evolution of the *Drosophila buzzatii* (Diptera: Drosophilidae) cluster using mtDNA. *Annals of the Entomological Society of America* **94**: 333–346.
- Manfrin MH, Sene FM. 2006. Cactophilic *Drosophila* in South America: a model for evolutionary studies. *Genetica* **126**: 57–75.
- Matzkin LM. 2014. Ecological genomics of host shifts in *Drosophila mojavensis*. In: Landry C, Aubin-Horth N, eds. *Ecological genomics. Advances in Experimental Medicine and Biology*, vol. 781. Dordrecht: Springer, 233–247.
- Menezes BF, Vigoder FM, Peixoto AA, Varaldi J, Bitner-Mathé BC. 2013. The influence of male wing shape on mating success in *Drosophila melanogaster*. *Animal Behaviour* **85**: 1217–1223.
- Meyer BN, Mohamed YA, McLaughlin JL. 1980.  $\beta$ -Phenethylamines from the cactus genus *Opuntia*. *Phytochemistry* **19**: 719–720.
- Moraes EM, Spressola VL, Prado PRR, Costa LF, Sene FM. 2004. Divergence in wing morphology among sibling species of the *Drosophila buzzatii* cluster. *Journal of Zoological Systematics and Evolutionary Research* **42**: 154–158.
- Morales-Hojas R, Vieira J. 2012. Phylogenetic patterns of geographical and ecological diversification in the subgenus *Drosophila*. *PLoS One* **7**: e49552.
- Muller K, Thiéry D, Delbac L, Moreau J. 2016. Mating patterns of the European grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae) in sympatric and allopatric populations. *Biological Journal of the Linnean Society* **120**: 685–699.
- Oliveira DC, Almeida FC, O'Grady PM, Armella MA, DeSalle R, Etges WJ. 2012. Monophyly, divergence times, and evolution of host plant use inferred from a revised phylogeny of the *Drosophila* repleta species group. *Molecular Phylogenetics and Evolution* **64**: 533–544.
- Olson DM, Dinerstein E, Wikramanayake ED, Burgess ND, Powell GV, Underwood EC, Loucks CJ, D'amico JA, Itoua I, Strand HE, Morrison JC, Loucks CJ, Allnutt TF, Ricketts TH, Kura Y, Lamoreux JF, Wettengel WW, Hedao P, Kassem KR. 2001. Terrestrial ecoregions of the world: a new map of life on Earth: a new global map of terrestrial ecoregions provides an innovative tool for conserving biodiversity. *BioScience* **51**: 933–938.
- Padró J, Carreira V, Corio C, Hasson E, Soto IM. 2014. Host alkaloids differentially affect developmental stability and wing vein canalization in cactophilic *Drosophila buzzatii*. *Journal of Evolutionary Biology* **27**: 2781–2797.
- Pigliucci M. 2003. Phenotypic integration: studying the ecology and evolution of complex phenotypes. *Ecology Letters* **6**: 265–272.
- Renaut S, Bernatchez L. 2011. Transcriptome-wide signature of hybrid breakdown associated with intrinsic reproductive isolation in lake whitefish species pairs (*Coregonus* spp. Salmonidae). *Heredity* **106**: 1003–1011.
- Reti L, Castrillón JA. 1951. Cactus alkaloids. I. *Trichocereus terscheckii* (Parmentier) Britton and Rose. *Journal of the American Chemical Society* **73**: 1767–1769.
- Richmond MP, Johnson S, Markow TA. 2012. Evolution of reproductive morphology among recently diverged taxa in the *Drosophila mojavensis* species cluster. *Ecology and Evolution* **2**: 397–408.
- Rieseberg LH, Archer MA, Wayne RK. 1999. Transgressive segregation, adaptation and speciation. *Heredity* **83**: 363–372.
- Rohlf FJ. 2015. The tps series of software. *Hystrix, the Italian Journal of Mammalogy* **26**: 9–12.
- Sackton TB, Haney RA, Rand DM. 2003. Cytonuclear coadaptation in *Drosophila*: disruption of cytochrome c oxidase activity in backcross genotypes. *Evolution* **57**: 2315–2325.
- Schluter D. 2001. Ecology and the origin of species. *Trends in Ecology & Evolution* **16**: 372–380.
- Soto EM, Mongiardino Koch N, Milla Carmona P, Soto IM, Hasson E. 2017. Cactus–fungi interactions mediate host preference in cactophilic *Drosophila* (Diptera: Drosophilidae). *Biological Journal of the Linnean Society* **122**: 539–548.
- Soto EM, Soto IM, Carreira VP, Fanara JJ, Hasson E. 2008. Host-related life history traits in interspecific hybrids of cactophilic *Drosophila*. *Entomologia Experimentalis et Applicata* **126**: 18–27.
- Soto IM, Carreira VP, Corio C, Padró J, Soto EM, Hasson E. 2014. Differences in tolerance to host cactus alkaloids in *Drosophila koepferae* and *D. buzzatii*. *PLoS One* **9**: e88370.
- Soto IM, Carreira VP, Soto EM, Hasson E. 2008. Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*. *Journal of Evolutionary Biology* **21**: 598–609.
- Soto IM, Manfrin MH, Sene F, Hasson E. 2007. Viability and developmental time in the cactophilic *Drosophila gouveai* and *D. antonietae* (Diptera, Drosophilidae) are dependent of the cactus host. *Annals of the Entomological Society of America* **4**: 490–496.
- Stintzing FC, Carle R. 2005. Cactus stems (*Opuntia* spp.): a review on their chemistry, technology, and uses. *Molecular Nutrition & Food Research* **49**: 175–194.
- Thomas-Orillard, M. 1984. Modifications of mean ovariole number, fresh weight of adult females and developmental time in *Drosophila melanogaster* induced by *Drosophila C* virus. *Genetics* **107**: 635–644.
- Tidon-Sklorz R, Sene FM. 2001. Two new species of the *Drosophila serido* sibling set (Diptera, Drosophilidae). *Iheringia Serie Zoologia* **90**:141–146.
- Valente LM, Scheinvar LA, da Silva GC, Antunes AP, Dos Santos FA, Oliveira TF, Tappin MRR, Neto FRA, Pereira AS, Carvalhaes SF, Siani AC, Soares ROA, Ferreira EF, Bozza M, Stutz C, Gibaldi D, Dos Santos RR. 2007. PHCOG MAG.: Research Article Evaluation of the antitumor and trypanocidal activities and alkaloid profile in species of Brazilian Cactaceae. *Pharmacognosy Magazine* **3**: 167.

- Wagner GP, Laubichler MD. 2004.** Rupert Riedl and the re-synthesis of evolutionary and developmental biology: body plans and evolvability. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* **302**: 92–102.
- Wilson K, Hardy IC. 2002.** Statistical analysis of sex ratios: an introduction. In: Hardy I, ed. *Sex ratios: concepts and research methods*. Cambridge: Cambridge University press, 48–92.
- Wu CI, Davis AW. 1993.** Evolution of postmating reproductive isolation: the composite nature of Haldane's rule and its genetic bases. *The American Naturalist* **142**: 187–212.
- Zar JH. 1996.** *Biostatistical analysis*. Upper Saddle River: Prentice Hall Inc.

### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Box plots, showing sex ratios (males/females + males) for the different crossing types.

**Fig. S2.** Path analysis diagram of direct and indirect correlations between development time, larval viability and wing size on overall fitness.

**Table S1.** Parameter coefficients for the generation means model.

**Table S2.** ANOVA results for fitness traits of *D. koepferae*, *D. antonietae* and their interspecific hybrids reared in prickly pears and columnar cactus hosts.