Evolutionary Biology
https://doi.org/10.1007/s11692-017-9441-8

RESEARCH ARTICLE

Experimental Evolution of Alkaloid Tolerance in Sibling *Drosophila* Species with Different Degrees of Specialization

Julián Padró[1,2] · Diego N. De Panis[1] · Juan Vrdoljak[1,3] · Pablo Milla Carmona[1,4] · Betina Colines[1] · Esteban Hasson[1] · Ignacio M. Soto[1]

Received: 17 April 2017 / Accepted: 25 November 2017

Abstract

Drosophila buzzatii and *Drosophila koepferae* are sibling species with marked ecological differences related to their patterns of host exploitation. *D. buzzatii* is a polyphagous species with a sub-cosmopolitan distribution, while *D. koepferae* is endemic to the mountain plateaus of the Andes, where it exploits alkaloidiferous columnar cacti as primary hosts. We use experimental evolution to study the phenotypic response of these cactophilic *Drosophila* when confronting directional selection to cactus chemical defenses for 20 generations. Flies adapted to cactus diets also experienced higher viability on alkaloid-enriched media, suggesting the selection of adaptive genetic variation for chemical-stress tolerance. The more generalist species *D. buzzatii* showed a rapid adaptive response to moderate levels of secondary metabolites, whereas the columnar cacti specialist *D. koepferae* tended to maximize fitness under harder conditions. The evolutionary dynamic of fitness-related traits suggested the implication of metabolic efficiency as a key mediator in the adaptive response to chemical stress. Although we found no evidence of adaptation costs accompanying specialization, our results suggest the involvement of compensatory evolution. Overall, our study proposes that differential adaptation to secondary metabolites may contribute to varying degrees of host specialization, favoring niche partitioning among these closely related species.

Keywords Adaptation · Secondary metabolites · Hormesis · Cactus · Chemical stress · Specialization

Introduction

Ecological specialization has been a focal interest for many generations of biologists who have noted that increasing biodiversity is accompanied by niche diversification, enabling the coexistence of multiple species. In this sense, differential habitat use has been associated with morphological, physiological and behavioral diversity, providing the raw materials for interspecific divergence and speciation processes (Futuyma and Moreno 1988; Jablonski 2017). Within terrestrial ecosystems, the radiation of phytophagous insects is one of the best examples of this phenomenon. Among the factors shaping patterns of insect-plant interactions, chemical defenses deployed by plants are salient features. In effect, secondary metabolites have long been identified as one of the most remarkable factors driving the evolutionary dynamics of host-plant specialization (Ehrlich and Raven 1964; Rosenthal and Berenbaum 2012; Agrawal and Weber 2015). Because of their ecological role, these molecules are classified as "allelochemicals", a term referring to non-nutritional chemicals produced by individuals of a species

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11692-017-9441-8) contains supplementary material, which is available to authorized users.

✉ Julián Padró
padrojulian@comahue-conicet.gob.ar

[1] Instituto de Ecología, Genética y Evolución de Buenos Aires (IEGEBA – CONICET). DEGE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Int. Guiraldes 2160, Buenos Aires, Argentina

[2] Present Address: Ecotono Laboratory, INIBIOMA (CONICET-National University of Comahue), Quintral 1250, CP 8400 Bariloche, Argentina

[3] Present Address: Instituto Patagónico para el Estudio de los Ecosistemas Continentales, Consejo Nacional de Investigaciones Científicas y Técnicas (IPEEC-CONICET), Boulevard Almirante Brown 2915, U9120ACD Puerto Madryn, Chubut, Argentina

[4] Laboratorio de Ecosistemas Marinos Fósiles, Instituto de Estudios Andinos Don Pablo Groeber (CONICET-UBA), Intendente Güiraldes 2160, Ciudad Universitaria (C1428 EHA), Buenos Aires, Argentina

Published online: 06 December 2017

that affects the physiology of another (Whittaker and Feeny 1971). As a consequence, evolution of chemical-stress buffering mechanisms is expected to be a main target of natural selection affecting insects' diet range (Brattsten et al. 1977; Gloss et al. 2014). In fact, most of the evidence supporting the role of adaptive plastic responses to host-plants refers to induced expression of detoxification genes and life-history traits (Nylin and Janz 2009).

Although there has been considerable interest in the study of mechanisms driving specialization to host-plants and the accompanying genetic changes, the processes leading to "generalism" has received little attention (Loxdale et al. 2011). The general postulate is that specialists should be more tolerant to secondary metabolites, although the incurred metabolic cost of carrying built-in detoxification mechanisms predicts a loss in the ability to use multiple host-plants, restricting the range of suitable niches. In contrast, generalists should retain more general and less costly buffering mechanisms, allowing wider ecological niches, with potentially larger distribution ranges (Loxdale et al. 2011; Ali and Agrawal 2012). Hence, predictability of levels and types of allelochemicals should also be a key factor driving metabolic strategies of host-plant adaptation (Agrawal 2001). In this context, the harsh lifestyle of desert *Drosophila* associated with cacti provides an excellent opportunity for studying adaptation to chemical stress and its implications in divergent evolution (e.g., Barker and Starmer 1982; Fogleman and Danielson 2001; McGirr et al. 2017).

The use of necrotic cacti as breeding sites in South American cactophilic *Drosophila* has linked their evolutionary history to the distributional fluctuations and diversification of Cactaceae during climate changes caused by the Quaternary glacial periods (Manfrin and Sene 2006; Franco and Manfrin 2013). Recent comparative studies of patterns of host-plant use in the *D. buzzatii* cluster showed that the use of *Opuntia* is common throughout the phylogeny and that shifts to alkaloidiferous columnar cacti occurred several times independently (Oliveira et al. 2012). Moreover, some specialists in rearing on columnar cacti have lost the use of *Opuntia* even as secondary hosts, highlighting the potential role of ecological specialization during phylogenetic radiation.

Patterns of host-plant use in the cluster *D. buzzatii* has been thoroughly described in the sibling pair of species *D. buzzatii* and *D. koepferae* (Hasson et al. 2009). Their varying degree of specialization to host cacti is a main manifestation of their genetic divergence (Fanara et al. 1999; Fanara and Hasson 2001; Piccinali et al. 2004, 2007). *D. buzzatii* breeds primarily on the necrotic stems of prickly pears (genus *Opuntia*) but displays a more generalist habit since it can be recovered from rotting pockets of many species of columnar cacti (genera *Cereus, Trichocereus, Praecereus, Pilosocereus*) and has even been observed to emerge from commercial fruits (Hasson et al. 1992; Camargo et al. 2016; Fanara et al. 2016). Originally from southern South America, in the last 200 years *D. buzzatii* has reached a sub-cosmopolitan distribution colonizing Europe, Australia and Africa (Barker 2013). In contrast, *D. koepferae* is restricted to the highland deserts of northwestern Argentina and southern Bolivia, where its primary host is the columnar cactus *Trichocereus terscheckii* and secondarily may use *Opuntia* as a less preferred host (Hasson et al. 1992). The strict co-occurrence of *D. koepferae* and *T. terscheckii* in Argentina suggests a certain degree of specialization that may limit the dispersal range.

Recent studies provided evidence that phenylethylamine alkaloids of columnar cactus *T. terscheckii* are a stress factor for larval development (with *D. buzzatii* being markedly more sensitive than *D. koepferae*), yet it still remains unclear whether alkaloids are key determinants in cactus specialization (Soto et al. 2014). Differences among species in tolerance to allelochemicals may arise as a cause or consequence of habitat use. Varying degrees of specialization among species may lie in habitat/host-plant fidelity, as well as in the diet/tolerance range (Futuyma and Moreno 1988; Loxdale et al. 2011). Thus, it is also uncertain whether these species have the potential to adapt to variable levels of the defense chemistry imposed by columnar cacti.

We designed a protocol to investigate the genetic basis and phenotypic consequences of long-term chemical-stress exposure in these closed related species with divergent evolutionary strategies. First, we design a full-sib experiment to study the relative contribution of genetic variation, phenotypic plasticity and genotype by environment interaction (G × E) of fitness-related traits across a gradient of cactus allelochemicals. We predict higher levels of genetic variation and phenotypic plasticity in the more generalist species *D. buzzatii*, but lower levels of G × E interaction to varying level of allelochemicals, compared to the specialist *D. koepferae*. Secondly, we performed artificial selection to varying doses of cactus allelochemicals to compare the adaptive potential and phenotypic response of both species. We anticipate a faster adaptation to higher levels of allelochemicals in *D. koepferae* than its sibling *D. buzzatii*, but with similar phenotypic responses due their shared evolutionary history. Third, we evaluated the role of alkaloids as major determinants of cactus adaptation and examined the costs of specialization to different levels of chemical-stress. We predict that selected lines would evolved greater tolerance to alkaloids than Control lines, and we also expect a higher adaptation cost in *D.buzzatii* as a more generalist species.

Materials and Methods

Collection of Stocks

Flies were collected following the methodologies proposed by Markow and O'Grady (2005), using entomological nets and fermented banana traps. Wild populations were sampled in ten locations of northwestern Argentina during the austral summer of 2013 (Online Resource 1). The area encompasses two major biogeographic units, the Monte desert rich in columnar cacti of the genus *Trichocereus*, and the dry Chaco where cacti are mostly represented by the genus *Opuntia* (Cabrera 1976). Flies of both species were collected in the Monte region, along with stems of *Trichocereus terscheckii*, (that were stored at − 20 °C until the onset of the experiments), whereas in the Chaco region, *D. buzzatii* was the only species found. The progeny of wild-caught inseminated females were used to establish isofemale lines. Since these two species are morphocryptic, the identification was performed by examining the male genitalia of F1s for each isofemale line (Soto et al. 2007). A total of 46 isofemale lines of *D. koepferae* and 43 of *D. buzzatii* were established and maintained under controlled conditions (12:12 h light/dark photoperiod at 25 ± 1 °C) in our standard rearing medium (150 g starch, 0.3 g $CaCl_2$, 0.3 g KCl, 0.2 g $MgSO_4$, hydrated with a protein solution: 40 g yeast extract, 25 mL ethanol, 0.25 mL propionic acid, and 1.8 g Nipagin per 1 L water). For the experimental treatments described below we extracted tissue rich in allelochemicals from stems of *T. terscheckii*: the layer of chlorenchyma was removed, homogenized in a blender, dehydrated (78hs oven-dry at 50 °C), sieved to a fine powder and stored dry. Collection permits were issued by the Secretariat of Environment and Sustainable Development of San Juan province (File N° 1300-0236-13).

Experimental Design

Preliminary Experiments (Genetic Basis)

Before the onset of the selection protocol, and in order to test the reaction norm of different genotypes to increasing concentration of cactus (Fig. 1), we employed an experimental design including homokaryotypic strains (inbred for eight generations), derived from ten isofemale lines, carrying the three most frequent second chromosome inversions found in *D. buzzatii* (*standard, j* and *jz3*) and *D. koepferae* ($2l^9$, $2\ m^9$ and $2n^9$; Fontdevila et al. 1988; Fernández Iriarte and Hasson 2000). Batches of 40 instar larvae of each isofemale line were seeded in vials (5 replicates per line) containing 1 gram of rearing media. Larvae were exposed to 4 treatments that differed in the proportions of each rearing medium (treatments: 100/0, 75/25, 50/50 and 25/75% standard/chlorenchyma media, respectively) hydrated with 5 mL of the protein solution (described above). We measured larval viability as a direct component of early fitness, thus a good estimator of adaptation to host cactus chemistry: cacti tissue constitutes the most immediate environment affecting early life-cycle. Additionally, we measured developmental time and wing size. These set of traits, apparently evolved as adaptations to exploit host plants, are related with adult fitness components such as reproductive success, dispersion,

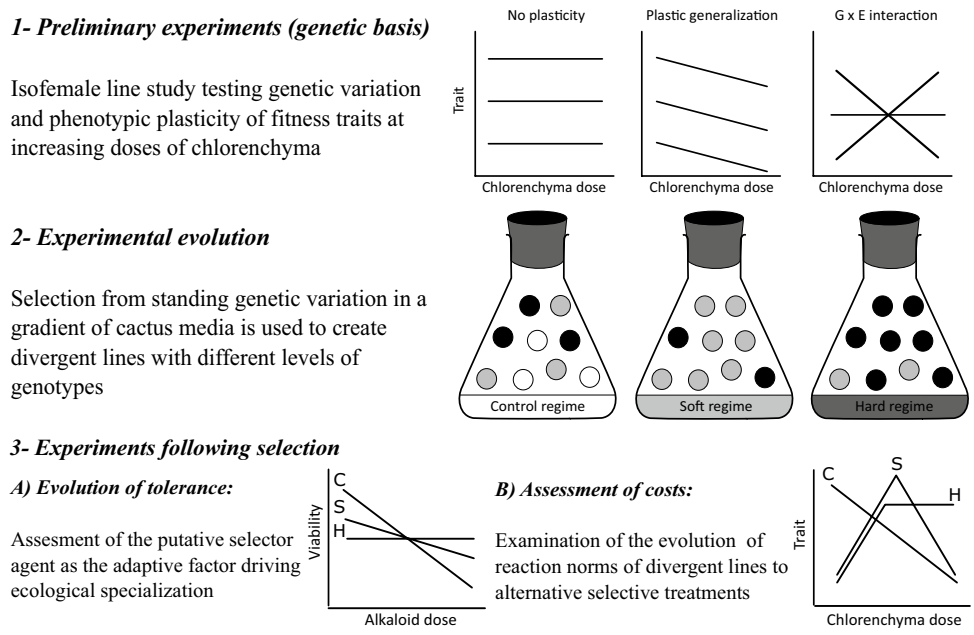

Fig. 1 Schematic representation of the experimental design

longevity, stress tolerance, and are accurate indicators of environmental quality in *Drosophila* (e.g., Santos et al. 1988; Sgro and Hoffmann 2004; Soto et al. 2014). Prior to statistical analyses viability and developmental time scores were angularly and Log transformed, respectively, to comply with the assumptions of the statistical tests (Zar 1996).

For wing size measurements, the right wing of 460 and 281 male adults of *D. buzzatii* and *D. koepferae*, respectively, were mounted on slides and digitalized to place ten representative landmarks (details in Padró et al. 2014). Wing size was estimated through the centroid size (square root of the sum of the squared distance of each landmark to the geometric centroid of the landmarks), using TpsDIG2 (Rohlf 2015).

We employed analysis of genotypic adaptation in multi-environment trials (Cooper et al. 1996), using the following two-way ANOVA model for each trait:

$$Y = \mu + G + E + G \times E + \text{Error},$$

where μ is the overall mean effect, G the genotypic random effect (genetic differences among lines), E the environment fixed effect (generalized phenotypic plasticity), while G × E estimate Genotype by Environment effects. Since G × E may arise due to (Eq. 1) heterogeneity of genotypic variance across treatments (changes in scale) and/or (Eq. 2) lack of genetic correlation across environments (changes in rank order), we assessed the contribution of these two sources. The proportional relative contribution was estimated from the variance components of the two-way ANOVA model previously described and the genotypic variance components were estimated for each environment through separate one-way ANOVAs (model: Y = μ + g + Error) according to the formulas (Annicchiarico 2002):

$$V\left(\delta_{g(j)}\right)/\delta^2_{(G\times E)} \quad (1)$$

$$\left(\delta^2_{(G\times E)} - V\left(\delta_{g(j)}\right)\right)/\delta^2_{(G\times E)} \quad (2)$$

where $\delta^2_{(G\times E)}$ corresponds to the variance components of G × E in the two-way ANOVA model and $V(\delta_{g(j)})$ the variance of the square root values of genotypic variance components estimated through one-way ANOVAs in each environment *j*.

In addition, we calculated the pooled genetic correlation among environments as an estimator of the relative size of G × E that is not due to heterogeneity of genotypic variance:

$$r_g = \delta^2_{(G)}/\left(\delta^2_{(G)} + \delta^2_{(G\times E)} - V\left(\delta_{g(j)}\right)\right)$$

where $\delta^2_{(G)}$ stands for the genotypic variance components in the two-way ANOVA model. Thus, r_g values ranges from zero to unity, indicating the degree of predictability in the genotypic response to increasing cactus concentrations.

Experimental Evolution

Base populations of each species were generated by mixing equal numbers of flies from the established isofemale lines (total of ~2000 adults per species) to maximize the amount of genetic variation. Given that isofemale lines of *D. buzzatii* were originally collected from different biogeographic units which differ in the presence of its secondary host *T. terscheckii*, before merging into the mass population, we first confirmed that there were no differences in larvae performance when exposed to the experimental treatments detailed below (Online Resource 2). These stocks were maintained by mass breeding for three generations before the onset of the experiment. Batches of ~200 mated females of these populations were randomly distributed in 9 cages that were exposed to Control (C), Soft (S) or Hard (H) selection regimes (3 replicate populations per regime). C populations were reared in 100% standard medium, S populations in 50/50% standard/chlorenchyma powder medium and H populations in 25/75%, respectively. Experimental populations were maintained under controlled uncrowded larval conditions in discrete generations. In each generation, 200 virgins of each population (replicates) were transferred to a common oviposition chamber for no longer than 72hs to start the next generation. Every 5 generations for *D. buzzatii* and in generations #0, #6, #13 and #20 for *D. koepferae*, we assessed changes in viability, developmental time and wing size of each population in their respective experimental regime. The experimental design (Fig. 1) was carried out in the same way as described in preliminary experiments (total analyzed wings: 1151 for *D. buzzatii* and 761 for *D. koepferae*). To estimate the evolutionary dynamics of the populations exposed to different regimes, we employed partial quadratic regression analyses on phenotypic values, assessing linear and non-linear selection gradients (Lande and Arnold 1983). Differences in evolutionary profiles among regimes were analyzed *via* repeated-measures analyses (multivariate approach) with factors "Regime", "Generation" and the interaction "Regime by Generation" (populations as replicates). Statistical analyses were performed using GLM repeated-measures ANOVA procedure as implemented in STATISTICA (StatSoft, Tulsa, OK, USA).

Experiments Following Selection

Evolution of Tolerance After 20 generations of experimental evolution, we assessed changes in the tolerance to chemical stress by measuring larval viability in each experimental population exposed to increasing doses of purified alkaloids isolated from *T. terscheckii* (Fig. 1; extraction protocol is described in De Panis et al. 2016). The obtained alkaloid fraction, composed mostly of mescaline and trichocereine, was added to the rearing media in three different concen-

trations: 10 mg/g (natural concentration), 20 mg/g and 30 mg/g. Larvae of each experimental population were seeded in vials containing 1 g of standard medium with the addition of the different doses of alkaloids. To exclude acclimation effects on the responses, three generations of relaxed selection in standard medium were applied to all experimental populations prior to trials. Statistical analyses of stress tolerance of the experimental lines were performed by means of linear regressions of viability (dependent variable) on the increasing doses of pure alkaloids (independent factor). Additionally, we tested homogeneity of slopes between selected and Control lines to evaluate the evolution of sensitivity to alkaloids in each species.

Assessment of Costs In order to assess whether selection regimes elicited changes in the reaction norm of experimental populations, we performed a "reciprocal transplant" experiment by assessing samples of all regimes in each of the treatments (Fig. 1; Kassen 2002; Conner 2003; Murren et al. 2014). Adults were allowed to lay eggs in Petri dishes and batches of first-instar larvae were seeded in vials containing C, S or H media. We measured viability and developmental time in all populations, as these traits showed to be highly sensitive to semi-natural media. Reaction norm analyses were performed applying the following two-way ANOVA model: $Y = \mu + R + E + R \times E + \text{Error}$, where R stands for the fixed effect of each regime, which estimates the overall difference among lines across all environments (mean elevation of the reaction norm), E is the fixed environmental effect of the chlorenchyma media across all lines, while R x E is the interaction effect, reflecting differential responses of experimental lines across environments. In addition, we assessed changes in other attributes of the reaction norm such as the slope (S) and curvature (C), using phenotypic values (Z) of each trait according to equations (Murren et al. 2014):

$$S = \frac{\sum_1^{n-1} S_i}{n-1}; \text{ where } S_i = Z_{i+1} - Z_i$$

$$C = \frac{\sum_1^{n-2} C_i}{n-2}; \text{ where } C_i = S_{i+1} - S_i$$

where n indicates the number of chlorenchyma doses and i the focal dose (treatment). Analyses on the computed scores S and C (dependent variables) were performed by means of one-way ANOVAs for each trait (model: $Y = \mu + R + \text{Error}$) to test differences across selection regimes.

Results

Quantitative Genetic Analysis

Increased amounts of cactus chlorenchyma elicited a reduced viability and wing size, while extending developmental time in both species, although *D. buzzatii* was markedly more sensitive than its sibling (Online Resources 3 and 4). The general ANOVAs showed further differences for the specific traits in each species. G × E interaction was significant for all traits in both species, except for viability in *D. buzzatii* which exhibited a generalized plastic response (Table 1). While in *D. koepferae* the interaction was mostly explained by changes in the rank order of genotypes, in *D. buzzatii* both components of G × E had a fairly balanced contribution. Moreover, genotypic correlation analyses revealed a consistent response of genotypes across environments for wing size in *D. koepferae* and developmental time in *D. buzzatii*. Overall, our results denoted that phenotypic variation has both genetic and environmental bases, though species showed distinctive contributions in their components of genotypic variation.

Table 1 Variance components and quantitative genetic statistics for fitness traits in *D. buzzatii* and *D. koepferae* exposed to increasing cactus concentrations

	V_g	V_e	$V_{(G\times E)}$	$r_{(G\times E)}$	$V_{(G\times E)\ \text{partitioned}}$
D. koepferae					
Viability	3.143**	NS	1.579**	0.703	16.1–83.9
Developmental time	0.041*	***	0.040***	0.549	15.7–84.3
Wingsize	0.170***	NS	0.005*	0.971	4.3–95.7
D. buzzatii					
Viability	8.829***	***	NS	N/A	N/A
Developmental time	0.015**	***	0.005***	0.851	44.1–55.9
Wingsize	NS	**	0.063***	0.448	33.3–66.7

V_g denotes among-line effects indicating genetic variation. V_e are fixed effects of environments denoting phenotypic plasticity. $V_{(G\times E)}$ accounts for line by environment interaction. $r_{(G\times E)}$ is the pooled genetic correlation among environments. $V_{(G\times E)}$ partitioned are the proportions of genotype by environment interaction attributable to heterogeneity of genotypic variance (first term) and changes in the rank order among environments (second term)

N/A not applicable, *NS* non-significant

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Evolution of Fitness Components

Viability

Repeated measures analysis revealed significant differences among the viability profiles of the experimental regimes (Table 2). The analysis across generations showed that larval viability remained fairly stable along the experiment in *D. koepferae* S populations, whereas C and H populations showed opposite trends: after the sixth generation Control populations exhibited a steady and linear reduction, whereas H lines increased viability at a rate of almost 5% per generation (Table 3; Fig. 2a). In contrast, *D. buzzatii* H populations exhibited a linear and steady drop of viability at a rate of 3.4% per generation. A similar initial trend was observed in *D. buzzatii* S populations, however these lines were able to evolved higher viabilities, even 13% more than in the initial generation (Fig. 2b).

Table 2 Repeated measures analyses (*F*-values) testing for differences among regimes, generational time and its interaction for fitness traits

Factor/trait	Df^n_d	Viability	Dev. time	Wing size
D. buzzatii				
Time × regime	$^8_{24}$	7.05***	2.49*	3.42**
Time	$^4_{24}$	7.50***	3.15*	7.77***
Regime	$^2_6$	6.29*	185.67***	19.61**
D. koepferae				
Time × regime	$^6_{18}$	4.30**	3.36*	1.08
Time	$^3_{18}$	0.69	18.04***	1.47
Regime	$^2_6$	6.62*	92.45***	17.36**

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

Developmental Time

The evolutionary dynamic of developmental time was significantly different among regimes (Table 2). *D. koepferae* S and H populations evolved extended developmental time, but at different rates: S lines escalated linearly at a rate of 0.4% per generation (Table 3) whereas H lines reached a *plateau* after six generations of constant increase (Fig. 2c). *D. buzzatii* S populations also increased developmental time, at a mean rate of 0.8% per generation, reaching values similar to H lines. During the initial generation of exposure to the H regime, the base population extended developmental time, although it remained steady along generational time (H lines, Fig. 2d). Despite Control lines of *D. koepferae* displayed a significant regression, the magnitude of the variation was very low, while in *D. buzzatii* C lines remained steady (see Fig. 2c, d).

Wing Size

Wing development was differentially affected by rearing conditions in each species (Table 2). Wing size of *D. koepferae* lines remained fairly steady across time (Table 3; Fig. 2e). In contrast, S and H populations of *D. buzzatii* followed a non-linear selection gradient (after an initial size reduction), reaching values similar to in Control populations (Fig. 2f).

Analysis of Chemical-Stress Tolerance

Regression analysis revealed a notable decline in larval viability as alkaloid concentration increased in *D. buzzatii* Control populations, with a maximum reduction of 65% at the highest dose ($F_{1,13} = 4.30$, $P = 0.05$, $\beta = -0.08$, $r^2 = 0.25$). Control lines of *D. koepferae* exhibited a 16% reduction, though the regression was not significant ($F_{1,13} = 0.11$, $P = 0.74$), indicating an intrinsic developmental

Table 3 Summary of polynomial regressions testing for linear (β), and non-linear (γ) selection gradients on fitness components for each regime

	Viability			Development time			Wing size		
	Control	Soft	Hard	Control	Soft	Hard	Control	Soft	Hard
D. koepferae									
F-value (df = 2,9)	4.23†	NS	7.81*	6.65*	7.42*	7.90*	NS	NS	NS
Lineal term β_1	− 0.02*	N/A	−0.02*	NS	0.01**	L/F	N/A	N/A	N/A
Quadratic term γ	< 0.01†	N/A	< 0.01†	< −0.01**	NS	L/F	N/A	N/A	N/A
D. buzzatii									
F-value (df = 2,12)	NS	4.28*	15.45***	NS	8.05**	NS	NS	9.73**	4.13*
Lineal term β_1	N/A	NS	− 0.01***	N/A	0.01**	N/A	N/A	− 0.01*	L/F
Quadratic term γ	N/A	< 0.01*	NS	N/A	NS	N/A	N/A	< 0.01**	L/F

N/A not applicable, *NS* non-significant, *L/F* lack of fit

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

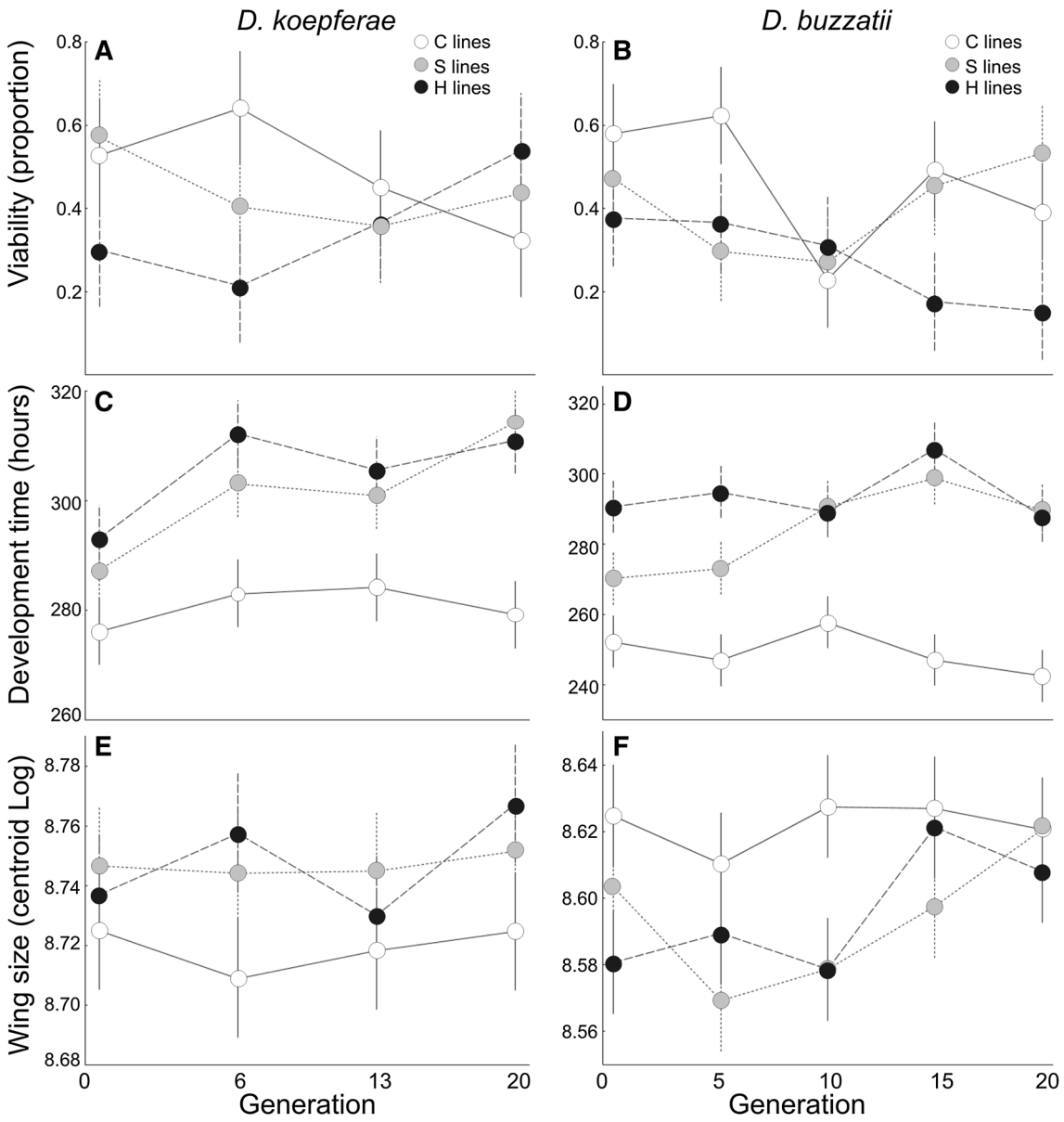

Fig. 2 Experimental evolution of viability (**a, b**), developmental time (**c, d**) and wing size (**e, f**) in *D. koepferae* and *D. buzzatii* Control, Soft and Hard selection regimes. Bars denote 95% confidence interval

robustness in the presence of alkaloids. Analyses performed in selected populations of both species revealed no significant regressions ($P > 0.05$, in all cases). Overall, the evolution of alkaloid tolerance in selected lines was mostly related to higher mean viability levels in both species (Fig. 3a, b). However, the homogeneity of slope comparisons performed between selected and Control lines was marginally significant in *D. buzzatii* H lines, indicating a decreased sensitivity to alkaloids effects ($F_{1,26} = 4.0$; $P = 0.05$).

Evaluation of Reaction Norms

Results of significant changes in the components of the reaction norms among experimental lines are shown in Table 4. *D. buzzatii* S and H populations diverged mainly in the elevation of the reaction norm for both viability and developmental time. In the case of *D. koepferae* the response of these traits in selected populations differed not only due to changes in mean elevation, but also because of significant curvature variation (Table 4). In general, selected populations of both species tended to achieve higher mean

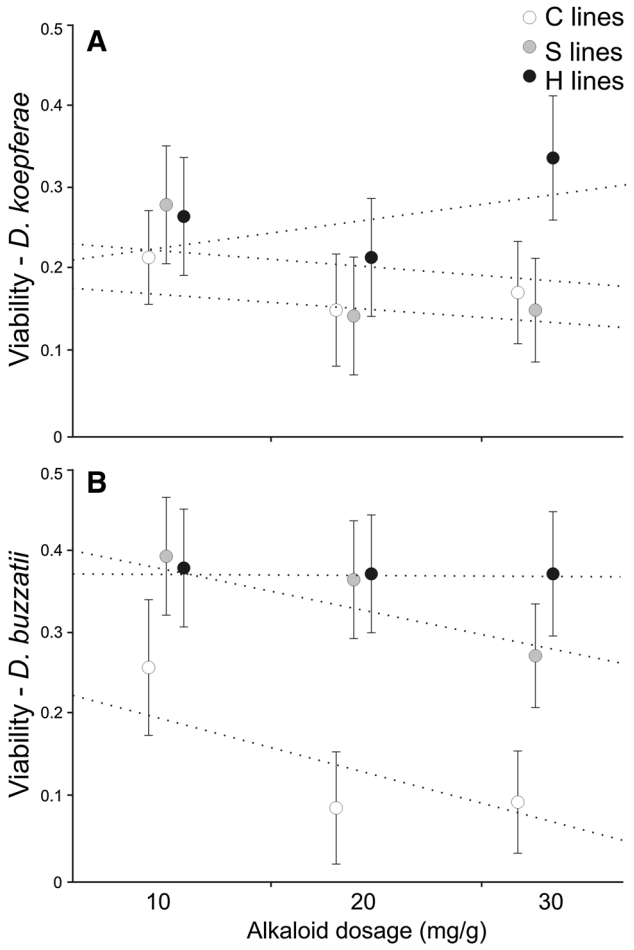

Fig. 3 Larval viability along increasing doses of alkaloids in experimental populations of *D. koepferae* (**a**) and *D. buzzatii* (**b**). Standard deviation bars and linear trends are shown

Table 4 Summary of analysis of reaction norms (*F*-values), testing for differences among experimental regimes (mean elevation), environmental effect, parallel responses among environments (interaction term), slopes and curvatures for life history traits

Factor/trait	df^{n}_{d}	Viability	Dev. time
D. koepferae			
Regime (elevation)	$^{2}_{12}$	23.90***	16.96***
Environment	$^{2}_{12}$	7.03**	111.32***
Regime × environment	$^{4}_{12}$	7.18**	6.58**
Slope	$^{2}_{6}$	1.95	4.15
Curvature	$^{2}_{6}$	7.64*	5.0*
D. buzzatii			
Regime (elevation)	$^{2}_{12}$	8.74**	196.27***
Environment	$^{2}_{12}$	25.19***	70.60***
Regime × environment	$^{4}_{12}$	0.73	3.00
Slope	$^{2}_{6}$	1.97	1.5
Curvature	$^{2}_{6}$	0.18	4.03

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

viabilities and longer developmental times than Controls, although differences were only consistently apparent in *D. buzzatii* development times (Fig. 4a–d). In addition, phenotypic values of selected lines raised in standard medium (0% chlorenchyma) were significantly different from Control values, indicating that the response to selection was not due to an acclimation effect, except in *D. koepferae* S lines which reverted to the Control phenotype (i.e., not differ from Control values; Fig. 4a, c).

Discussion

Genetic Basis of Fitness Traits

Even in a small heterogenic subsample of genotypes we found substantial genetic variation in fitness related-traits of both species (Table 1), which at least are partially heritable (Cortese et al. 2002). These results suggest that the stock of isofemale lines of both species possessed sufficient selectable variation (G×E) for populations to evolve under different doses of chlorenchyma. In addition, phenotypic responses of fitness-related traits revealed that increasing doses of chlorenchyma compromised viability, extended developmental time and reduced wing size (Online Resources 3 and 4), suggesting an adaptive plastic response through an increased detoxification metabolism at the expenses of growth (Hoffmann and Parsons 1989; Padró et al. 2014). These results are in agreement with previous studies that investigated the detrimental effects produced by *T. terscheckii* (Soto et al. 2008; Corio et al. 2013), validating the use of the cactus chlorenchyma powder in selection experiments. The present results also provide some relevant points to interpret the outcome of selection experiments in the light of these species' ecology. First, the effect of rearing media on fitness traits was species-specific. As may be expected by patterns of host plant use in nature, *D. buzzatii* was more sensitive to *T. terscheckii* chlorenchyma, (Fanara and Hasson 2001; Soto et al. 2008, 2014). Moreover, the differential contribution of the genetic components of larval viability among species suggests different adaptive strategies to cope with cactus chemical defenses. *D. buzzatii* showed plastic generalization (i.e., parallel norms of reaction) while significant G×E interaction in *D. koepferae* indicated the presence of varying degrees of specialized genotypes. The second aspect arising from the study is that the relative contribution of the components of G×E interactions of developmental time and wing size differed among species (Table 1). This implies not only that components of genetic variation diverged between species, but also suggests that different genes/traits might be involved depending on the concentration of cactus, enabling the evolution of different phenotypes (Clark and Fucito 1998).

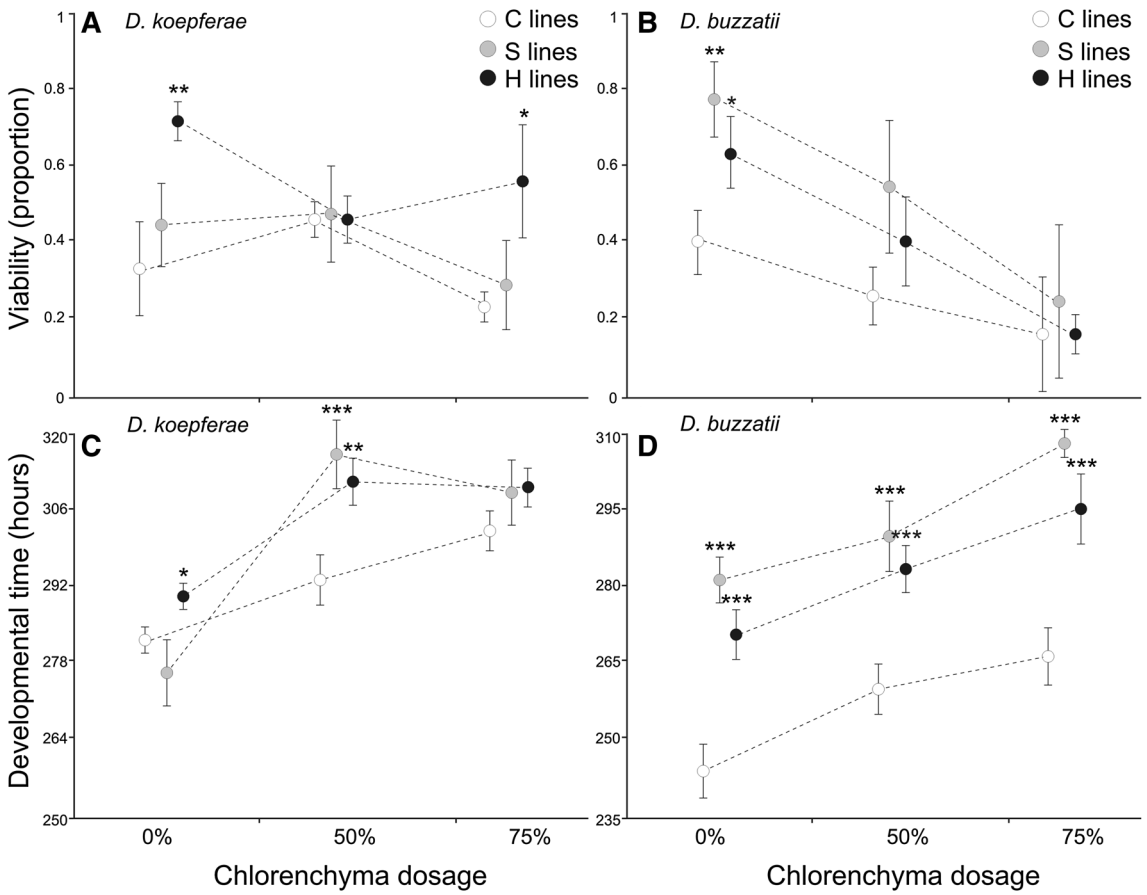

Fig. 4 Reaction norms of life history traits of experimental lines reared in media with different concentrations of chlorenchyma. Significant differences of selected lines relative to the Control in each environment are shown. Dunnet test: $*P<0.05$; $**P<0.01$; $***P<0.001$

Evolution of Fitness

Our experiment showed that despite components of genetic variation have diverged since the last common ancestor, both species were able to respond to selection by increasing larval viability in cactus media. However, the evolutionary dynamics depended on the species-specific degrees of specialization to cactus hosts in nature. While the cactophilic generalist *D. buzzatii* increased mean viability in the moderate regime, the columnar specialist *D. koepferae* optimized performance in the most extreme environment (Table 3; Fig. 2a, b). The observed pattern of maximum fitness at different levels of allelochemicals is consistent with an evolutionary process of stress-derived hormesis (Forbes 2000). Hormesis refers to a generalizable phenomenon in organisms dealing with dose–response relationships: the invigorating effects of low doses of toxins that are harmful at higher levels (Calabrese and Baldwin 2003). Processes associated with hormesis have been related with the colonizing ability in many species of *Drosophila*, including the role of ethanol in the habitat use of *D. melanogaster* (Parsons 2001; Costantini et al. 2010). Given that *D. koepferae* and *D. buzzatii* are species with a differential use of stressful habitats, the displacement of the hormetic zone (chemical range where phytotoxins produce beneficial effects) is an evolutionary expectation with ecological implications in niche partitioning. In this context, the response of non-linear size increase with extended developmental time in selected lines (Fig. 2c–f) could be reflecting the phenotypic byproducts of metabolic efficiency selection to varying levels of secondary metabolites (Parsons 2001; Padró et al. 2014). In contrast, Control populations of both species showed a faster developmental time and larger wing sizes (except in *D. koepferae*) than selected lines (Table 3; Fig. 2c–f), suggesting a relaxed regime, favorable for larval development (Soto et al. 2014). Thus, the noticeable evolution of larval viability and developmental time found in Control lines of *D. koepferae* could be explained as the consequence of an environmental range outside the hormetic zone in a species adapted to extreme levels of secondary metabolites.

Evolution of Alkaloids Tolerance

Experimental populations selected in cactus media also exhibited the greatest mean viability when exposed to purified alkaloids (Fig. 3), supporting the hypothesis of genetic adaptation to alkaloid stress as a direct result of natural selection. This is in line with many studies performed in other desert *Drosophila*, where a rapid evolution of increased tolerance to alkaloids was found to be mediated by detoxification enzymes (Fogleman and Danielson 2001; Bono et al. 2008; Matzkin 2012). Moreover, the implication of induced expression of the exogenous metabolism has been related with energy efficiency, affecting organism performance, development control and ecological dynamics (Marden 2013). In addition, the regression analysis of viability on alkaloid concentrations in Control populations reinforced the idea that *D. koepferae* is endowed with more robust detoxification mechanisms than *D. buzzatii*, allowing a more effective exploitation of the alkaloidiferous *T. terscheckii* (Soto et al. 2014). Recent analysis of differential gene expression revealed a genome wide transcriptional plasticity involving a vast functional diversity when *D. buzzatii* is reared in *T. terscheckii* with increased levels of alkaloids. For instance, P-450 genes involved in detoxification, hormones metabolism and development regulation (directly related to developmental time) are implicated in hormetic mechanisms and appeared to be pivotal in the complex response of *D. buzzatii* to alkaloid exposure (Timbrel 2009; De Panis et al. 2016). Thus, differences in the evolutionary dynamics among species might be reflecting the divergent evolution of highly pleiotropic genes (i.e., affecting multiple traits) responsible for alkaloid stress tolerance. Nonetheless, further comparative studies of the transcriptomic responses between *D. buzzatii* and *D. koepferae* will allow us to further dissect the genomics of host adaptation.

Adaptation Costs

Theoretical models of phenotypic evolution predicts adaptation costs as a consequence of specialization (Futuyma and Moreno 1988; Lande 2009), however we did not find evidence indicative of associated costs. Selected populations shifted the elevation of the reaction norm of viability and developmental time, revealing remarkable changes in larval metabolism, with no directional effects in environmental sensitivity across cactus concentrations (i.e., slope; Table 4). These results indicate no evidence of *trade-offs* or a significant reduction in environmental sensitivity as result of adaptation costs. In fact, contrary to the idea of a fitness cost imposed by carrying resistant alleles in the absence of xenobiotics, selected populations tended to increase larval viability when raised in standard medium (Fig. 4a, b), suggesting that adaptation to chemical stressors might not be necessarily costly (Wan et al. 2017). A possible explanation could rely on the fact that adaptation to alkaloids may arise due to many causes: efficiency of detoxification mechanisms, sequestration of metabolites, energy expenditure, resource allocation, interaction with nutrient absorption and interferences with general biological processes (Wink et al. 1998; Mithöfer and Boland 2012). In effect, experiments of resource acquisition (a core feature of specialization) performed in *Drosophila* frequently results in a lack of evidence supporting antagonist pleiotropy due to specialization and even yielded positive correlations (Kolss et al. 2009; Vijendravarma et al. 2012; Goenaga et al. 2013). Thus, complex interactions among genetic/physiological pathways underlying resource exploitation might mask *trade-offs* when they actually occur. In our case, the evolution of increased wing size (significant in *D. buzzatii;* Table 3), a trait associated with mating success in *Drosophila*, suggests the implication of compensatory evolution of fitness-enhancing genes (Santos et al. 1988; McKenzie and Clarke 1988; Pischedda and Chippindale 2005).

Concluding Remarks

Overall, our study points to the idea that host shifts can contribute to diversification in closely related species through metabolic adaptation to the chemical environment. In the face of an ecological challenge, as overcoming chemical defenses of novel host-plants, the maintenance of fitness could depend on the evolution of stress-derived hormesis. Our results suggested a displaced hormetic zone among species as a putative consequence of different degrees of host specialization. This scenario could help to explain why generalist insects usually succeed in various groups of plants with mild defenses, while specialists frequently thrive in chemically challenging hosts. Although the study of the genetic architecture underlying hormetic mechanisms still lacks empirical investigation, the level of integration between exogenous and endogenous metabolism seems a promising approach to disentangle the complex evolution of adaptive plasticity.

Acknowledgements We want to thanks to Pedro Fontanarrosa for assistance in running the experiments, and to Sergio Szajnman for invaluable technical support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Agrawal, A. A. (2001). Phenotypic plasticity in the interactions and evolution of species. *Science, 294*(5541), 321–326.

Agrawal, A. A., & Weber, M. G. (2015). On the study of plant defence and herbivory using comparative approaches: How important are secondary plant compounds. *Ecology Letters, 18*(10), 985–991.

Ali, J. G., & Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. *Trends in Plant Science, 17*(5), 293–302.

Annicchiarico, P. (2002). *Genotype × environment interaction: Challenges and opportunities for plant breeding and cultivar recommendations*. Rome: Food and Agriculture Organization of the United Nations.

Barker, J. S. F. (2013). Genetic history of a colonizing population: *Drosophila buzzatii* (Diptera: Drosophilidae) in Australia. *Biological Journal of the Linnean Society, 109*(3), 682–698.

Barker, J. S. F., & Starmer, W. T. (1982). *Ecological genetics and evolution: The cactus-yeast-Drosophila model system*. London: Academic Press.

Bono, J. M., Matzkin, L. M., Castrezana, S., & Markow, T. A. (2008). Molecular evolution and population genetics of two *Drosophila mettleri* cytochrome P450 genes involved in host plant utilization. *Molecular Ecology, 17*(13), 3211–3221.

Brattsten, L. B., Wilkinson, C. F., & Eisner, T. (1977). Herbivore-plant interactions: Mixed-function oxidases and secondary plant substances. *Science, 196*(4296), 1349–1352.

Cabrera, A. L. (1976). *Enciclopedia Argentina de Agricultura y Jardinería*. Buenos Aires: ACME.

Calabrese, E. J., & Baldwin, L. A. (2003). Toxicology rethinks its central belief. *Nature, 421*(6924), 691–692.

Camargo, F. P., Araujo, A. C. V., de Moraes, E. M., & Dos Santos, A. C. A. (2016). A comparison between cactophilic yeast communities isolated from *Cereus hildmannianus* and *Praecereus euchlorus* necrotic cladodes. *Fungal Biology, 120*(10), 1175–1183.

Clark, A. G., & Fucito, C. D. (1998). Stress tolerance and metabolic response to stress in *Drosophila melanogaster. Heredity, 81*(5), 514–527.

Conner, J. K. (2003). Artificial selection: A powerful tool for ecologists. *Ecology, 84*(7), 1650–1660.

Cooper, M., DeLacy, I. H., & Basford, K. E. (1996). Relationships among analytical methods used to analyse genotypic adaptation in multi-environment trials. In M. Cooper, G.L. Hammer (Eds.), *Plant adaptation and crop improvement* (pp. 193–224). Wallingford: CAB International in association with IRRI and ICRISAT.

Corio, C., Soto, I. M., Carreira, V., Padró, J., Betti, M. I., & 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*(2), 342–353.

Cortese, M. D., Norry, F. M., Piccinali, R., & Hasson, E. (2002). Direct and correlated responses to artificial selection on developmental time and wing length in *Drosophila buzzatii. Evolution, 56*(12), 2541–2547.

Costantini, D., Metcalfe, N. B., & Monaghan, P. (2010). Ecological processes in a hormetic framework. *Ecology Letters, 13*(11), 1435–1447.

De Panis, D. N., Padró, J., Furió-Tarí, P., Tarazona, S., Carmona, M., Soto, P. S., I. M., … & Hasson, E. (2016). Transcriptome modulation during host shift is driven by secondary metabolites in desert *Drosophila. Molecular Ecology, 25*(18), 4534–4550.

Ehrlich, P. R., & Raven, P. H. (1964). Butterflies and plants: A study in coevolution. *Evolution, 18*, 586–608.

Fanara, J. J., Fontdevila, A., & Hasson, E. (1999). Oviposition preference and life history traits in cactophilic *Drosophila koepferae* and *D. buzzatii* in association with their natural hosts. *Evolutionary Ecology, 13*(2), 173–190.

Fanara, J. J., & Hasson, E. (2001). Oviposition acceptance and fecundity schedule in the cactophilic sibling species *Drosophila buzzatii* and *D. koepferae* on their natural hosts. *Evolution, 55*(12), 2615–2619.

Fanara, J. J., Soto, I. M., Lipko, P., & Hasson, E.. (Patterson (2016). First record of *Drosophila buzzatii* & Wheeler) (Diptera: Drosophilidae) emerging from a non-cactus host. *Neotropical Entomology, 45*(3), 333–335.

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*(4), 1295–1302.

Fogleman, J. C., & Danielson, P. B. (2001). Chemical interactions in the cactus-microorganism-*Drosophila* model system of the Sonoran Desert 1. *American Zoologist, 41*(4), 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*(3), 380–385.

Forbes, V. E. (2000). Is hormesis an evolutionary expectation? *Functional Ecology, 14*(1), 12–24.

Franco, F. F., & Manfrin, M. H. (2013). Recent demographic history of cactophilic *Drosophila* species can be related to Quaternary palaeoclimatic changes in South America. *Journal of Biogeography, 40*(1), 142–154.

Futuyma, D. J., & Moreno, G. (1988). The evolution of ecological specialization. *Annual Review of Ecology and Systematics, 19*(1), 207–233.

Gloss, A. D., Vassão, D. G., Hailey, A. L., Dittrich, A. C. N., Schramm, K., Reichelt, M., … & Montfort, W. R. (2014). Evolution in an ancient detoxification pathway is coupled with a transition to herbivory in the Drosophilidae. *Molecular Biology and Evolution, 31*(9), 2441–2456.

Goenaga, J., Fanara, J. J., & Hasson, E. (2013). Latitudinal variation in starvation resistance is explained by lipid content in natural populations of *Drosophila melanogaster. Evolutionary Biology, 40*(4), 601–612.

Hasson, E., Naveira, H., & Fontdevila, A. (1992). The breeding sites of Argentinian cactophilic species of the Drosophila mulleri complex (subgenus *Drosophila*-repleta group). *Revista chilena de historia natural, 65*(3), 319–326.

Hasson, E., Soto, I. M., Carreira, V. P., Corio, C., Soto, E. M., & Betti, M. (2009). .Host plants, fitness and developmental instability in a guild of cactophilic species of the genus *Drosophila*. In *Ecotoxicology research developments*. New York: Nova Science Publisher Inc.

Hoffmann, A. A., & Parsons, P. A. (1989). An integrated approach to environmental stress tolerance and life-history variation: Desiccation tolerance in *Drosophila. Biological Journal of the Linnean Society, 37*(1-2), 117–136.

Jablonski, D. (2017). Approaches to macroevolution: 2. Sorting of variation, some overarching issues, and general conclusions. *Evolutionary Biology, 44*, 451–475.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology, 15*(2), 173–190.

Kolss, M., Vijendravarma, R. K., Schwaller, G., & Kawecki, T. J. (2009). Life-history consequences of adaptation to larval nutritional stress in *drosophila. Evolution, 63*(9), 2389–2401.

Lande, R. (2009). Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *Journal of Evolutionary biology, 22*(7), 1435–1446.

Lande, R., & Arnold, S. J. (1983). The measurement of selection on correlated characters. *Evolution, 37*, 1210–1226.

Loxdale, H. D., Lushai, G., & Harvey, J. A. (2011). The evolutionary improbability of 'generalism'in nature, with special reference to insects. *Biological Journal of the Linnean Society, 103*(1), 1–18.

Manfrin, M. H., & Sene, F. M. (2006). Cactophilic *Drosophila* in South America: A model for evolutionary studies. *Genetica, 126*(1–2), 57–75.

Marden, J. H. (2013). Nature's inordinate fondness for metabolic enzymes: Why metabolic enzyme loci are so frequently targets of selection. *Molecular Ecology, 22*(23), 5743–5764.

Markow, T. A., & O'Grady, P. M. (2005). *Drosophila*:. In *a guide to species identification and use*. New York: Academic Press.

Matzkin, L. M. (2012). Population transcriptomics of cactus host shifts in *Drosophila mojavensis. Molecular Ecology, 21*(10), 2428–2439.

McGirr, J. A., Johnson, L. M., Kelly, W., Markow, T. A., & Bono, J. M. (2017). Reproductive Isolation Among *Drosophila arizonae* from Geographically Isolated Regions of North America. *Evolutionary Biology, 44*(1), 82–90.

McKenzie, J. A., & Clarke, G. M. (1988). Diazinon resistance, fluctuating asymmetry and fitness in the Australian sheep blowfly, *lucilia cuprina. Genetics, 120*(1), 213–220.

Mithöfer, A., & Boland, W. (2012). Plant defense against herbivores: Chemical aspects. *Annual Review of Plant Biology, 63*, 431–450.

Murren, C. J., Maclean, H. J., Diamond, S. E., Steiner, U. K., Heskel, M. A., Handelsman, C. A., … & Relyea, R. A. (2014). Evolutionary change in continuous reaction norms. *The American Naturalist, 183*(4), 453–467.

Nylin, S., & Janz, N. (2009). Butterfly host plant range: An example of plasticity as a promoter of speciation? *Evolutionary Ecology, 23*(1), 137–146.

Oliveira, D. C., Almeida, F. C., O'Grady, P. M., Armella, M. A., DeSalle, R., & Etges, W. J. (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*(3), 533–544.

Padró, J., Carreira, V., Corio, C., Hasson, E., & Soto, I. M. (2014). Host alkaloids differentially affect developmental stability and wing vein canalization in cactophilic *Drosophila buzzatii. Journal of Evolutionary Biology, 27*(12), 2781–2797.

Parsons, P. A. (2001). The hormetic zone: An ecological and evolutionary perspective based upon habitat characteristics and fitness selection. *The Quarterly Review of Biology, 76*(4), 459–467.

Piccinali, R., Aguadé, M., & Hasson, E. (2004). Comparative molecular population genetics of the Xdh locus in the cactophilic sibling species *Drosophila buzzatii and D. koepferae. Molecular Biology and Evolution, 21*(1), 141–152.

Piccinali, R. V., Mascord, L. J., Barker, J. S. F., Oakeshott, J. G., & Hasson, E. (2007). Molecular population genetics of the α-Esterase5 gene locus in original and colonized populations of *Drosophila buzzatii* and its sibling *Drosophila koepferae. Journal of Molecular Evolution, 64*(2), 158–170.

Pischedda, A., & Chippindale, A. (2005). Sex, mutation and fitness: Asymmetric costs and routes to recovery through compensatory evolution. *Journal of Evolutionary Biology, 18*(4), 1115–1122.

Rohlf, F. J. (2015). The tps series of software. *Hystrix, The Italian Journal of Mammalogy, 26*(1), 9–12.

Rosenthal, G. A., & Berenbaum, M. R.(2012). *Herbivores: Their interactions with secondary plant metabolites: Ecological and Evolutionary Processes* (Vol. 2). London: Academic Press.

Santos, M., Ruiz, A., Barbadilla, A., Quezada-Díaz, J. E., Hasson, E., & Fontdevila, A. (1988). The evolutionary history of *Drosophila buzzatii*. XlV. Larger flies mate more often in nature. *Heredity, 61*, 255–262.

Sgro, C. M., & Hoffmann, A. A. (2004). Genetic correlations, tradeoffs and environmental variation. *Heredity, 93*(3), 241–248.

Soto, I. M., Carreira, V. P., Corio, C., Padró, J., Soto, E. M., & Hasson, E. (2014). Differences in tolerance to host cactus alkaloids in *Drosophila koepferae* and *D. buzzatii*. PLoS ONE, 9(2), e88370.

Soto, I. M., Carreira, V. P., Fanara, J. J., & Hasson, E. (2007). Evolution of male genitalia: Environmental and genetic factors affect genital morphology in two *Drosophila* sibling species and their hybrids. *BMC Evolutionary Biology, 7*(1), 77.

Soto, I. M., Carreira, V. P., Soto, E. M., & Hasson, E. (2008). Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila. Journal of Evolutionary Biology, 21*(2), 598–609.

Timbrel, J. A. (2009). *Principles of biochemical toxicology* (4th edn.). London: Informa Healthcare.

Vijendravarma, R. K., Narasimha, S., & Kawecki, T. J. (2012). Adaptation to abundant low quality food improves the ability to compete for limited rich food in *Drosophila melanogaster*. PLoS ONE, 7(1), e30650.

Wan, J. S., Pang, C. K., & Bonser, S. P. (2017). Does the cost of adaptation to extremely stressful environments diminish over time? A literature synthesis on how plants adapt to heavy metals and pesticides. *Evolutionary Biology, 44*, 411–426.

Whittaker, R. H., & Feeny, P. P. (1971). Allelochemicals: Chemical interactions between species. *Science, 171*(3973), 757–770.

Wink, M., Schmeller, T., & Latz-Brüning, B. (1998). Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA, and other molecular targets. *Journal of Chemical Ecology, 24*(11), 1881–1937.

Zar, J. H. (1996). *Biostatistical analysis*. New Jersey: Prentice Hall Inc.