Host plant adaptation in cactophilic species of the *Drosophila buzzatii* cluster: fitness and transcriptomics

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ABSTRACT

Host plant shifts in herbivorous insects often involve facing new environments that may speed up the evolution of oviposition behavior, performance related traits, morphology and, incidentally, reproductive isolation. In the genus *Drosophila*, cactophilic species of the *repleta* group include emblematic species in the study of the evolution of host plant utilization. The South American *D. buzzatii* and its sibling *D. koepferae* are a model system for the study of differential host plant use. Though these species exhibit a certain degree of niche overlap, the former breeds primarily on decaying cladodes of *Opuntia* cacti while the *D. koepferae* main hosts are columnar cacti of the genus *Trichocereus*. *Opuntia sulphurea* and *T. terscheckii* are the among the main hosts in nature. These cacti differ in ecological (spatial and temporal predictability) and chemical characteristics. Particularly relevant is the presence of toxic alkaloids in *T. terscheckii*. Studies of the effects of these cacti and alkaloids revealed the remarkable impact on oviposition behavior, viability, developmental time, wing morphology, mating success and developmental stability in both species. Recent whole-genome expression studies showed that expression profiles are massively affected by the rearing cactus, and that the presence of alkaloids is the main factor modulating gene expression in *D. buzzatii*. Functional enrichment analysis indicated that differentially expressed genes are related to detoxification processes and stress response; though genes involved in development are an important part of the transcriptomic response. The implications of our studies in the evolution of host plant use in the *repleta* group are discussed.

KW: Host plant shifts-alkaloids-differential gene expression-developmental instability-mating success

INTRODUCTION

More than a half of recent insect families appeared in the Cretaceous, a biological revolution that is related either as cause or effect with the radiation of Angiosperms 130 Ma ago (Grimaldi & Engel 2005). Insects managed to exploit the new niches opened by the radiation of the Angiosperms and diversified. Furthermore, about half of the species of the major insect orders are phytophagous. Two pieces of evidence support the hypothesis that herbivory increases speciation rates: greater species richness in specialist herbivores than in non-herbivore-related taxa, and the link between host shifts and speciation in several herbivorous insect groups (Funk et al 2006; Simon et al 2015; Wiens et al 2015).

Herbivorous insects are involved in close interactions with host-plants as they represent food resource, mating and oviposition sites (Schoonhoven et al 2005). Such intimacy involved the evolution of defenses to impede the assault of herbivores. Hence, the shift to a new host plant may entail the challenge to exploit a new food source, face chemically diverse environments (including potentially toxic compounds), new mating environments, parasitoids and a different microflora (Kircher, 1982; Fogleman and Abril 1990; Jaenike 1990; Via, 1990; Schoonhoven et al 2005; Futuyma & Agrawal 2009; Forister et al 2012; Vogel et al 2014). Therefore, host plant shifts are expected to accelerate the evolution of features associated to performance in new hosts, oviposition behavior and sensory systems like those involved in smell and taste and morphological changes (Dambroski *et al*., 2005; McBride, 2007; Tilmon 2008; Earley & Jones 2011; Comeault et al 2015). Furthermore, habitat or diet shifts have been shown to be positively correlated with reproductive isolation in several groups and in Lepidoptera and *Drosophila* among insects (Funk et al 2006; Nosil, 2012). Thus, adaptation to a new host plant may also cause, either as a direct

consequence or as a byproduct, the evolution of sexual isolation, highlighting the evolutionary role of host plant shifts in speciation.

Concerning the genetic determinants of host plant use we are just starting to understand the plant–insect interactions at the molecular level. Genomic and post-genomic studies are contributing new insights to our understanding of the different elements involved in the genetic responses of organisms to alternative host plants and to the identification of loci and genomic regions associated to adaptation to new host plants (reviewed in Vogel et al 2014; Simon et al 2015). Particularly relevant, in this context, is to understand how insects overcome plant defenses allowing the acquisition of new hosts (De Panis et al 2016).

Plants synthesize an ample array of secondary metabolites as protection against herbivore insects. Thus, either true herbivores and insects feeding on decaying plant tissues may face dramatically challenging environments during a host plant shift, driving rapid adaptive phenotypic and genetic change (Schoonhoven et al 2005; Vogel et al. 2014; Simon et al 2015).

Fruit flies of the genus *Drosophila* have been instrumental in several disciplines, from genetics and development to ecology and evolution (Markow & O'Grady 2007). *Drosophila melanogaster* is the best-known species and one of the most meticulously studied organism in the history of modern science. Research in this model organism prompted advances in several areas thanks to the vast array of genetic tools available for genetic manipulation and dissection of traits of interest (e.g. Mackay et al 2012). In addition, a well annotated genome and the availability of genomic data allows detailed population genomic studies (Casillas & Barbadilla 2017). However, *D. melanogaster* has

the caveat of a poorly known ecology, preventing the correlation of genomic patterns of variation and the relevant ecological factors (Goldman-Huertas et al. 2015; Matzkin, 2014).

The ecology of *Drosophila* is highly diversified, there are species that breed on flowers, on mushrooms, tree fluxes (reviewed in Markow & O´Grady 2008). Even though most members of the main subgenera *Sophophora* and *Drosophila* are not true herbivores since they breed on necrotic plant tissues and feed upon the microorganisms (mainly yeasts) associated to the decaying process, the host plant constitutes the most immediate environmental factor affecting early life cycle stages. Most *Sophophora* species breed in rotting fruits, while species in the subgenus *Drosophila* display a greater diversity of resource specializations (Markow & O´Grady 2008). In the subgenus *Sophophora*, there are single-host specialists, such as *D. orena*, *D. erecta*, *D. sechellia* and *D. yakuba mayottensis* (Earley & Jones 2011; Linz et al 2013; Yassin et al 2016; Comeault et al 2015). *Drosophila sechellia* breeds on the decaying fruits of *Morinda citrifolia* (noni), which are toxic to other species of the *D. melanogaster* subgroup (Earley & Jones 2011; Huang & Erezyilmaz 2015). Likewise, the recently discovered *D. yakuba mayottensis* not only exhibits preference and higher tolerance for noni fruits but also a striking convergence with *D. sechellia* in common genetic pathways driving parallel adaptation to the toxic octanoic acid that is prevalent in ripe fruit (Huang & Erezyilmaz 2015; Yassin et al 2016).

Within the subgenus *Drosophila*, very few species groups are as amenable for the study of the evolutionary significance of host plant utilization as the cactophilic flies of the *repleta* group. Cactophilic *Drosophila*, except for the nannoptera group, are members of the *repleta* group, which radiated in the New World because of the ability to utilize decaying cacti as feeding and breeding substrates (Wasserman 1982).

The *Drosophila*-cactus-yeast system has long been a model system for ecological genetic studies (Barker & Starmer 1982; Heed & Mangan, 1986; Barker et al. 1990; Fogleman & Danielson, 2001; Guillén et al 2015). In this context, the guild of species that inhabit the desert of Sonora is the best-known case-study of adaptation to host plants that, in certain cases, imply the exposition of flies to toxic chemical compounds (Heed & Mangan 1986; Newby & Etges 1998; Fogleman & Danielson, 2001; Oliveira et al 2012; Smith et al 2013; Etges 2014; Matzkin 2014; Date et al 2015; Crowley-Gall et al 2016; Coleman et al 2018; Hoang et al 2015). In South America, the *D. buzzatii* cluster recently emerged as an excellent model system for the study of ecological adaptation to alternative host plants, since its members exhibit different degrees of host plant specialization and are in different stages of divergence (Manfrin & Sene 2006; Hasson et al 2009).

THE EVOLUTIONARY HISTORY OF THE *D. buzzatii* CLUSTER

In Central and South America, one of the radiations of the *D. repleta* group gave rise to the *Drosophila buzzatii* complex that includes the clusters *D. buzzatii, D. martensis* and *D. stalkeri* (Ruiz & Wasserman 1993). The former is an ensemble of seven closely related cactophilic species, *D. buzzatii*, *D. koepferae*, *D. antonietae*, *D. serido*, *D. borborema*, *D. seriema* and *D. gouveai,* that inhabit the arid and semiarid lands of southern South America (Fig 1). All species are endemic to South America, except *D. buzzatii* that reached a semi-cosmopolitan distribution following man mediated dispersion of plants of the genus *Opuntia* (prickly pear) (Fontdevila 1989).

Though morphologically very similar, species of the *D. buzzatii* cluster can be differentiated by the size and shape of male genitalia and fixed and polymorphic chromosomal inversions. Considering male genitalia, *D. buzzatii* is highly differentiated from the other six species which are more alike and are grouped in the so-called *D. serido* sibling set (Manfrin and Sene 2006). Cytologically four main lineages can be recognized based on shared fixed inversions. Inversions *5g* and *2j*[9] are fixed in *D. buzzatii* and *D. koepferae*, respectively; *D. serido* and *D. antonietae* share inversion $2x^7$ and *D. borborema*, *D. seriema* and *D. gouveai* share inversion $2e^8$ (Ruiz et al 2000). However, neither genital morphology nor chromosomal inversions are useful to discern the basal relationships. Molecular phylogenetic studies generated debate since tree topologies depend on the kind of molecular marker used (e.g. Rodriguez-Trelles et al 2000; Manfrin & Sene 2006). Particularly controversial is the position of *D. koepferae*, since according to external morphology, DNA sequence data of the mtDNA gene Cytochrome oxidase I gene (COI) and a few nuclear genes, *D. koepferae* is *D. buzzatii*´s closest relative (Manfrin *et al* 2001; Oliveira et al 2012). Alternatively, *D. koepferae* appears as the most basal lineage within the *D. serido* sibling set considering male genital morphology (Tidon-Sklorz & Sene, 2001) and other molecular data (Rodriguez-Trelles et al 2000; Manfrin and Sene 2006; Oliveira et al 2012). Nevertheless, we recently proposed an alternative phylogenetic hypothesis based on ca 1,000 genes selected from a large transcriptomic dataset obtained for *D. antonietae*, *D. borborema*, *D. buzzatii* and *D. koepferae* and *D. mojavensis* as outgroup. To obtain the dataset, we selected orthologus genes present in all four species and in the outgroup. Second, we filtered out all genes showing high similarity to *D. melanogaster* protein coding genes to remove highly conserved sequences that would provide little information to the phylogenetic analyses (details are available upon request). In the resulting tree, *D.*

koepferae appears as the sister species of *D. antonietae*, whereas *D. buzzatii* and *D. borborema* (in this order) represent the most basal lineages (Hurtado, Almeida, Revale & Hasson unpublished results). Unfortunately, the remaining species of the *D. buzzatii* cluster, that inhabit Brazilian arid lands, could not be included in the phylogenetic study, because they are difficult to obtain and are not available in *Drosophila* repositories, . Anyhow, these results emphasize the need for more exhaustive sampling of both genes and species to solve the phylogeny of the group.

THE ECOLOGICAL SETTING: CACTUS HOSTS AND THE SAPROPHYTIC COMMUNITY

Patterns of host plant use in the *D. buzzatii* cluster are mainly known in *D. buzzatii* and its sibling *D. koepferae* (Hasson et al. 1992, Fanara et al. 1999, 2006), a pair of sibling species with partial overlapping distributions in the arid lands of north-western Argentina and southern Bolivia (Fontdevila *et al.*, 1988; Hasson *et al.*, 1992). In contrast, information about host plant use in the other members of the cluster is scarce and restricted to emergence records or inferences based on the co-distribution of flies and host plants (Manfrin & Sene 2006). Indirect evidence suggests that the other species, *D. antonietae*, *D. gouveai*, *D. seriema*, *D. borborema* and *D. serido*, are mainly columnar cactus dwellers, though the latter can also be recovered from necrotic cladodes of prickly pear (Manfrin & Sene 2006, Oliveira et al 2012).

Field studies in *D. buzzatii* and *D. koepferae* showed that the former breeds primarily on decaying cladodes of prickly pear of the genus *Opuntia* while *D. koepferae* breeds mainly on columnar cacti of the genera *Cereus, Trichocereus* and *Neoraimondia* (formerly

Neocardenasia). However, there is some niche overlap since both species can be recovered from the same rotting pockets in nature (Fontdevila et al 1988; Hasson *et al.,* 1992). In the very arid Monte phytogeographic region of Argentina where *D. buzzatii* and *D. koefperae* are sympatric, *O. sulphurea* and *Trichocereus terscheckii* (cardón) are their respective primary hosts (Hasson et al 1992).

The interaction between cactus chemistry and microorganisms, yeasts and bacteria, involved in the decaying process of cactus tissues determines the chemical characteristics of the rots that flies utilize as breeding sites. Recent studies of the saprophytic communities associated to the necroses of cardón and *O. sulphurea*, revealed the presence of eight different species (Mongiardino-Koch et al 2015). *Pichia cactophila*, *Sporopachydermia cereana ´australis´* and *Prototheca zopfii* are cosmopolitan and generalists in terms of the cactus hosts from which they have been recovered (Lachance et al, 1998; Starmer et al, 2006). *Dipodascus australiensis* and *Magnusiomyces spicifer* were less frequent in the isolates and far less abundant in cardón than in *O. sulphurea* (Mongiardino-Koch et al 2015). Finally, *Yarrowia deformans*, the filamentous fungi *Fusarium lunatum* and the basidiomycetous yeast *Cryptococcus terrestris* were reported for the first time as autochthonous to cactus necroses by Mongiardino-Koch et al (2015). The first two species were recovered from both cacti and the third only in *O. sulphurea*. *Fusarium lunatum* not only was the most abundant in the samples of rots of both species but also deserves special attention since it has been recently identified as one of the causal agents of the cladode spot disease in *O. ficus-indica* (Flores-Flores et al., 2013).

CHEMICAL AND NUTRITIONAL PROFILES OF THE CACTUS HOSTS

Studies of the nutritional composition revealed that water content and energetic value is greater in cardón than in the prickly pear, while protein content is very much alike. In contrast, *O. sulphurea* contains about 2.5 times as much carbohydrates and fat than cardón (Padró & Soto 2013; Carreira et al 2014). In general, the main fatty acids in both cacti were quite similar to those reported for other columnar cacti and prickly pear, however, a rare isomer of oleic acid was abundant in *T. terscheckii* (Padró & Soto 2013).

Another central aspect of the *Drosophila*-cactus-yeast model system is the chemical composition of cactus species. Using standard techniques, we isolated alkaloid rich fractions from fresh tissues of *T. terscheckii* and *O. sulphurea*. Gas chromatography–mass spectrometry of these fractions showed two major peaks in *T. terscheckii* compatible with mescaline and trichocereine (N,N dimethylmescaline), whereas proline derivative alkaloids similar to 4-hydroxyproline were detected in the *Opuntia sulphurea* fraction (DePanis et al 2016).

In the present report, we present an updated account of our studies on the effects of host plants that differ sharply in chemical composition on fitness related traits, morphology, developmental instability and gene expression in the *Drosophila*-cactus-yeast system in Argentina.

OVIPOSITION AND PERFORMANCE IN ALTERNATIVE CACTUS HOSTS

There are two phases during which differential host plant use can occur along the life cycle of an insect. The first is host plant selection, in which volatile compounds produced

by the host are used by the insect as cues to locate a suitable site to mate and lay eggs. The second phase involves the utilization of the host plant which depends on the ability of the insect to use the host plant as a substrate. During the latter chemical factors such as nutritional quality and/or toxic compounds determine the suitability of the host plant.

Concerning the first phase, field studies showed that *D. buzzatii* and *D. koepferae* are equally attracted to traps prepared either with decaying *O. sulphurea* or *T. terscheckii* in the very arid Monte phytogeographic region in Northwestern Argentina (Fanara et al 1999). The proportions of both species collected in cactus baits prepared either with *O. sulphurea* or *T. terscheckii* differed substantially from collections on fermented banana baits (3:7 and 1:9 *D. buzzatii*:*D. koepferae*, respectively) that were simultaneously set up in the same area of collection (Fig 2a). However, emergence records from cactus rots revealed a completely different picture, since the proportions of *D. buzzatii* recovered from *O. sulphurea* and *T. terscheckii* rots were 66% and 7%, respectively (Fig 2a). These results suggest that oviposition behavior (differential oviposition hypothesis) and/or performance (differential performance hypothesis) during development depends on the cactus hosts in both species.

OVIPOSITION BEHAVIOR

We explored oviposition behavior using two approaches, both aimed to measure oviposition acceptance, not preference, since flies were offered a single choice as oviposition stimulus. In the first set of experiments, large numbers of inseminated females of two outbred stocks (one of each species) were released in egg collecting chambers (in the same proportions observed in natural baits in our field attraction experiment) in which an oviposition medium, consisting either of fermented *T. terscheckii* or *O. sulphurea* was poured in a petri dish that was replaced daily. Eggs were collected along three consecutive

days, allowed to hatch and first-instar larvae seeded in vials in optimal density (to avoid density dependent competition) in vials containing laboratory medium (see Fanara et al 1999 for details). In the second approach we used isofemale lines to evaluate whether variation in acceptance behavior has a genetic basis, otherwise, the experimental design was the same as above.

First instar-larva to adult viability in the first set of experiments was high and the proportion of *D. buzzatii* emerged varied along a three-day collecting period (Fig 2b). The proportion of *D. buzzatii* was considerably lower than the expected 30% in vials started with eggs sampled from both *O. sulphurea* and *T. terscheckii* chambers, particularly in the latter. However, the proportion of *D. buzzatii* increased steadily in the next two days, especially in vials started with samples of eggs collected in *O. sulphurea* chambers, in which the proportion of *D. buzzatii* reached 70%, but only 20% in cardón vials.

The experiments based on the use of isofemale lines as experimental units revealed species-specific oviposition preferences and fecundity schedules that were similar to the results obtained in the experiments with outbred stocks. In effect, *D. buzzatii* preferred to lay eggs on prickly pear and laid a fairly constant amount of eggs along the sampling period. *Drosophila koepferae*, on the other hand, exhibited a greater acceptance for its primary host and an oviposition peak on the second day of sampling. Additionally, our study revealed not only that oviposition acceptance in both species depends on the substrate offered to the flies, pointing to plastic behavioral responses, but also a genetic basis for such plastic responses, as suggested by the significant among genotype variation in both species (Fanara & Hasson 2001).

LARVAL PERFORMANCE AND ADULT TRAITS

Several traits are used as indicators of an organism's fitness in nature (Stearns, 1992). The expression of fitness related traits, as for all phenotypic traits, is mediated by both genetic and environmental influences. We investigated the effect of alternative host plants on performance by means of the quantification of several life history related traits. In the present account, we present the results of experiments aimed to measure developmental time as representative of early life history traits and adult survival, reproductive success, ovariole number and wing morphology as predictors of adult fitness components.

Developmental time (DT) provides an indication of the degree an organism's physiological and genetic mechanisms are adapted to exploit different kinds of resources (i.e. the ability to extract nutrients and eliminate toxic compounds). Fast development is expected to increase fitness in two ways, either through its positive effect on survival under conditions of larval crowding or through its putative demographic advantage for early reproduction in expanding populations (Roff 2000).

We measured DT as the time elapsed since first-instar larvae were transferred to the vials until adult emergence in both species reared in *O. sulphurea* or *T. terscheckii* under optimal conditions (5 larvae per ml of medium at 25ºC and room humidity). As expected the rearing medium differentially affected DT in both species, *D. buzzatii* performed better (developed faster) in *O. sulphurea* and *D. koepferae* in *T. terscheckii* (Fig 3) (Fanara et al 1999; Soto et al 2012). In addition, we also demonstrated that host related DT plasticity has a genetic basis as indicated by the significant genotype (isofemale line) by environment interaction in both species (Fanara et al 2006; Soto et al 2008a).

Concerning the effect of alternative hosts on adult fitness, we evaluated the hypothesis that flies reared in a species' preferred host increase mating success relative to flies reared in the unpreferred host. To this end, we released individual males (reared in *O. sulphurea* or *T. terscheckii*) and five females (reared in *O. sulphurea* or *T. terscheckii*) in mating chambers and recorded the number of matings (used as a surrogate of mating success) achieved by each male along a 30 minutes interval (see Hurtado et al 2012 for details). The assay involved male and female rearing cactus as factors and was replicated several times for each factor level combination. The results show that males of each species reared in the respective preferred host had more chances to mate than males reared in the secondary host (Fig 4). Moreover, male mating success was maximized when both males and females grew up in each species' preferred host. Even though body size is known to be affected by the rearing cactus (see below) and, as in many other taxa including *D. buzzatii*, is correlated with male mating success (Norry et al 1995), further analysis using body size as covariate indicated that the latter is not a predictor of mating success (Hurtado et al 2012).

Ovariole number is known to be phenotypically plastic in response to environmental conditions during juvenile development, particularly by larval nutrition in *D. melanogaster* (e.g. Hodin & Riddiford 2000). Thus, we also evaluated the effect of the rearing cactus on the number of ovarioles in flies reared in preferred and un-preferred hosts. Our study revealed a significant (but weak) preference-performance pattern only in *D. buzzatii* but not in *D. koepferae*. In effect, the former showed a 10% increase in the number of ovarioles in females reared in *O. sulphurea*. In addition, ovariole number did not show clear-cut differences between species (Peluso et al 2016).

Another relevant trait that may be affected by larval diet is adult survival during periods of food deprivation. Food shortage commonly affects organisms in nature when resources are temporally and spatially unpredictable. The ability to survive periods of famine is known as starvation resistance (SR) and can be measured as the time elapsed since flies are exposed to a starvation diet until death (see Soto et al 2012 for details). We measured SR in flies reared in media prepared with *O. sulphurea* or *T. terscheckii* and fed as adults with instant *Drosophila* medium before exposure to the starvation diet (see Soto et al 2012 for details). The rearing cactus affected starvation resistance in both species, however, not entirely as expected. The most striking findings were that flies reared in *O. sulphurea* were more resistant than flies reared in cardón, irrespective of the species, and, that *D. buzzatii* outlived *D. koepferae*, irrespective of the cactus medium (Fig 5).

Drosophila wings are known to respond to environmental variation in complex ways, suggesting that the reaction norm may be part of an adaptive response (e.g. David et al 2005). Thus, wing morphology is an excellent system to investigate the genetic and environmental factors that induce changes during development. Therefore, we studied wing size phenotypic plasticity dependent of the cactus host.

Our studies indicated that the cactus rearing medium affects wing morphology, *D. koepferae*, irrespective of the rearing substrate, and flies raised in *O. sulphurea*, regardless of the species, had larger wings than *D. buzzatii* and flies reared in *T. terscheckii*, respectively (Figure 6a) (see Soto et al 2008b for details). Additionally, we detected substantial genetic variation underlying wing morphology either as among genotypes (isofemale lines) variation or as genotype by cactus interaction (Carreira et al 2006; Hasson et al 2009).

Finally, wings, as bilateral organs, offer the chance to evaluate departures from normal development (developmental instability) provoked by environmental stressors by measuring fluctuating asymmetry (FA, defined as non-directional departures from bilateral symmetry). FA has been promoted as a general indicator of environmental stress. Positive correlations between environmental stress and FA have been reported in a wide variety of animals and plants (reviewed in Polak 2003). Chemical factors, demographic, nutritional stress, and extreme temperatures are correlated with FA (reviewed in Hoffman & Woods 2003; Zakharov 2003; Hasson et al 2009). In *Drosophila*, many studies addressed the effect of rearing temperature (e.g. Santos et al 2006), and, interspecific hybridization on wing development using FA as indicator (e.g. Rego et al 2006; Carreira et al 2008).

We tested the hypothesis that rearing in the un-preferred host increases fluctuating asymmetry. We found differences in the degree of FA between flies reared in preferred and un-preferred hosts in both *D. buzzatii* and *D. koepferae*. FA tended to be greater in flies emerged in the un-preferred host in both species (Figure 6b), however, the trend was significant in *D. koepferae*, but marginally significant in *D. buzzatii* (Soto et al., 2008). Our a priori expectation was a more pronounced FA in *D. buzzatii* since the shift from the chemically benign *O. sulphurea* to the alkaloidiferous columnar *T. terschekii* may be considered as more stressful than the shift in the opposite direction in *D. koepferae*. However, the pattern observed is in agreement with previous studies showing that *D. koepferae* is usually less plastic than *D. buzzatii* for performance traits (Fanara et al 1999), wing morphology (Carreira et al 2006), oviposition preference (Soto et al 2012), ovariole number (Peluso et al 2016) and differential gene expression (De Panis et al 2016 and unpublished results).

In summary, despite a preference for oviposition in *Opuntia* cacti, the studies outlined above showed that a shift to *T. terscheckii* hinders fitness in *D. buzzatii* by decreasing viability, body size, starvation resistance, ovariole number, extending development and increasing developmental instability and the reverse is, in general (but not always), true in *D. koepferae* (Fanara et al. 1999; 2006; Fanara & Hasson 2001; Carreira et al 2006; Soto et al 2008a; Soto et al 2008b; Hasson et al 2009; Soto et al. 2012).

ALKALOIDS AND PERFORMANCE

In view of the broad effect that cactus hosts have on several aspects of the biology of the flies and the differences in chemical composition reported between the main cactus hosts (DePanis et al 2016), we conducted experiments aimed at establish whether the negative effects of *T. terscheckii* on *D. buzzatii* can be accounted for by the presence of alkaloids in the rearing medium.

Thus, we assessed the effects alkaloid fraction extracted from *T. terscheckii* on overall performance using an index that includes viability, developmental time and adult body size, and, FA. Flies were reared in vials containing media prepared with standard Drosophila Instant medium with three alkaloid treatments: i) vials in which enough of the alkaloid fraction was added to reach the natural concentration in fresh cactus tissues (1X) and ii) doses of alkaloids 50% (1.5X) and iii) 100% (2X) higher than in native cactus. The rationale of assessing increasing amounts of the alkaloid fraction was that alkaloid concentration may vary during the decaying process due to water evaporation in the rotting pockets (Meyer & Fogleman 1987). Simultaneously, we evaluated the effect of the non-

alkaloid fraction obtained along the protocol of alkaloid extraction, since columnar cacti may contain other secondary compounds such as triterpene glycosides, sterol diols and rare fatty acids (Fogleman & Heed 1989) that may affect flies. Actually, *T. terschekii* contains cis-vaccenic acid, a rare isomer of oleic acid that is absent in *O. sulphurea* (Padró & Soto 2013).

Only *D. buzzatii* was affected by the presence of alkaloids in the rearing medium as indicated by regression analyses that revealed a negative association between performance and alkaloid dose (mainly explained by a decrease in larval viability and an extension of development, without affecting wing size) (Figure 7). The non-alkaloid fraction did not affect performance in *D. buzzatii* nor in *D. koepferae* (Soto et al 2014).

Finally, we measured FA in flies recovered from the three alkaloid treatments described in the preceding paragraph. Using a segmented regression analyses testing for a threshold dose for FA in wing size revealed a biphasic function with an action threshold at the intermediate alkaloid concentration and located the break point close to the 1.5X concentration, with positive and negative slopes at concentrations below and above the break point, respectively (see Padró et al 2014 for details). These results suggest a nonlinear effect of alkaloids on developmental stability.

Incidentally, wing inspection led to the discovery of anomalies in the pattern of wing venation correlated with alkaloid dose. The incidence of such anomalies, particularly in the posterior cross vein, was nil in the control, and increased as a function of alkaloid concentration (Padró et al 2014).

Genome-wide expression studies using RNA-Seq allow the evaluation of the genetic responses, in terms of gene expression, that organisms deploy in alternative environments. This approach is allowing the identification of candidate genes or genetic pathways underlying ecologically relevant phenotypes in insects (reviewed in Vogel et al. 2014; Simon et al 2015) and, particularly, in ecologically amenable *Drosophila* species (e.g. Smith et al. 2013; Hoang et al. 2015).

Recently, we reported new data on the role of transcriptional plasticity in the response to alternative hosts along with the assessment of genome-wide responses to mescaline-like alkaloids (De Panis et al 2016). Using RNA-Seq, we investigated the transcriptomic responses of *D. buzzatti* larvae raised in preferred and un-preferred cacti and exposed to cactus media with mescaline-like alkaloids. The experimental procedure consisted in raising batches of larvae from the first to the third instar in media prepared with native *O. sulphurea* (0x alkaloids) or native *T. terscheckii* (1X alkaloids) and native *O. sulphurea* or *T. terscheckii* supplemented with amounts of cardón alkaloid fraction necessary to achieve a final alkaloid concentration twice its native concentration in the native host plant (2X alkaloids both). All media were supplemented with a killed yeast extract as protein source. Our experimental design included three isofemale lines as biological replicates (details of the experimental design and procedures in De Panis et al 2016).

Our results showed that transcriptional responses were mainly modulated by the presence and concentration (to a lesser extent) of the alkaloid fraction (Table 1). In effect, comparisons between cacti, native *O. sulphurea* (0x alkaloids) vs *T. terscheckii* (1x

alkaloids) and between native *O. sulphurea* vs *O. sulphurea* + alkaloids (2x) illustrate this point, as shown by the great numbers of differentially expressed genes (DE genes) relative to other comparisons. Most DE genes were over-expressed in the more stressful condition in each comparison (native *T. terscheckii* and *O. sulphurea* + alkaloids, respectively). Moreover, the low number of DE genes detected in the comparison between native *T. terscheckii* and *T. terscheckii* 2x indicates that the transcriptomic response to a higher alkaloid concentration involved only a small number of genes relative to native *T. terscheckii*.

We further characterize the transcriptomic responses by means of enrichment analyses of GO terms related to biological processes and molecular functions on the set of DE genes. We detected an enrichment of detox-related and stress response GO terms in the sets of DE genes in treatments in which alkaloids were present (either native *T. terscheckii* and both 2x alkaloid treatments). In fact, DE genes involved in detoxification are members of the alcohol dehydrogenase (ADH), esterase (EST), glutathion transferase (GST), cytochrome P450 (P450) and UDP-glycosyltransferases (UGT) gene families typical of detoxification processes and associated to host plant utilization in insects (Matzkin et al 2006; Matzkin 2012; Smith et al 2013; Heidel-Fischer & Vogel 2015; Hoang et al 2015). To visualize gene expression patterns, we employed a Multi-Dimensional Scaling (MDS) approach, based on the expression scores of each one of the 62 genes in each combination of biological replicate (lines) and treatments. MDS allows the reduction of expression patterns to three dimensions, facilitating visualization and interpretation of transcriptomic similarity across treatments and biological replicates. Inspection of the MDS revealed not only that treatments can be discerned, despite the apparent heterogeneity among lines within

treatments, but also the greater similarity between alkaloid containing treatments than between treatments sharing the same cactus host plant (Fig 8).

Concerning stress response, the results of the functional enrichment analyses suggests that the cellular stress response seems to be mainly mediated by the ubiquitin-proteasome pathway and other related processes (protein folding and transport, translational regulation and response to DNA damage).

Besides detox and stress response functions, we also found enrichment for genes related to the development of different body structures. Furthermore, other DE genes provide a link between detox-related and development-related pathways. Notably, among the set of differentially expressed genes we detected genes associated with wing development that provide a link that may help phenotypic plasticity in wing morphology, departures from bilateral symmetry and the anomalous venation patterning observed in flies reared in *T. terscheckii* and/or in media with high alkaloid content. Finally, the functional enrichment analysis also revealed GO terms involved in development that may contribute to unravel the underlying genetic basis of DT variation in *D. buzzatii* reported.

DISCUSSION

The general conclusion of our studies is the remarkable influence that cactus hosts impose on several features of the biology of *D. buzzatii* and *D. koepferae*. In these cactophilic *Drosophila*, performance traits have probably evolved as adaptations to exploit resources with different ecological (spatial and temporal predictability) and chemical (nutritional quality and toxicity) characteristics (Fanara et al 1999; Hasson et al 2009).

Indeed, the results of oviposition behavior (acceptance), developmental time, and general body size assays fit well with predictions based on spatial and temporal availability of each species primary resource in nature. Cardón rots are relatively less abundant and last longer, whereas rotting cladodes of *O. sulphurea* are more abundant and ephemeral (Fanara et al 1999). These observations suggest that *T. terschekii*, a large stemmed cactus, may offer a more stable (predictable) habitat for larvae, but less predictable for adults searching for new feeding and oviposition sites than do small stemmed cacti like the prickly pear *O. sulphurea*, as argued by Heed & Mangan (1986) for Sonoran Desert *Drosophila*. Thus, it may be argued that utilization of a long lasting and patchy host plant drove *D. koepferae* life history evolution towards a massive reproductive output upon identification of a suitable breeding site and towards a larger body size (Fanara et al 1999; Carreira et al 2006), which is related to dispersal ability in cactophilic Drosophila (Markow & Castrezana, 2000). In the case of *D. buzzatii*, the scattered reproductive effort along time and the shorter DT likely evolved as adaptations to avoid crowding and to fare well in an ephemerous and spatially abundant resource. Moreover, the patterns of variation observed for developmental time, mating success, ovariole number and FA support the idea that both species maximize performance in the respective preferred host cactus. However, starvation resistance and wing size are exceptions to this general trend (Soto et al 2012).

The experiments based on the isofemale line technique showed that phenotypic plasticity for most traits has a genetic basis, visualized either as variation among genotypes or as genotype by environment interactions. Overall, our studies show that both species perceive alternative cactus hosts as different patches of a heterogeneous environment. The evolutionary significance of these results is clear, since environmental heterogeneity plays a

relevant role in the maintenance of species diversity and genetic variation in nature and with regard of the role of host plant shifts in diversification.

Our recent investigations on cactus chemistry revealed that *T. terscheckii* phenyl-ethylamine alkaloids (mescaline and trichocerine) are less harmful to the resident *D. koepferae* than to the non-resident *D. buzzatii* (Corio et al 2013; Soto et al 2014; Padró et al 2014). These results give support to the hypothesis, based on field and laboratory studies, that xenobiotics produced by cardón (particularly alkaloids) absent in *O. sulphurea*, can explain the differential performance of *D. buzzatii* and *D. koepferae* in alternative hosts.

Thus, we may conclude that both species independently evolved unique sets of adaptations to live in the desert and that the presence of xenobiotics, particularly alkaloids, is a major determinant of host-plant specificity. In both the South American and North American *Drosophila*-cactus model systems, alkaloids isolated from columnar cacti received special attention because its well-known role as herbivore deterrents. The proline derivative isolated from *O. sulphurea*, similar to compounds isolated from *O. vulgaris*, does not have reported toxicity (Jiang et al. 2003). In contrast, the mescaline like alkaloids isolated from *T. terscheckii* can account for the poor performance of *D. buzzatii* in cardón. This type of alkaloids is well-known because of a psychomimetic compound that has been isolated from other species of the genus *Trichocereus* (e.g. *T. pachanoi* ´San Pedro´cactus) and from peyote (*Lophophora williamsii*). These cacti have hallucinogenic properties and have been used in religious and therapeutic practices since ancient times. The type of alkaloids isolated from *Trichocereus* cacti is different from those in cacti used as breeding sites by Sonoran Desert *Drosophila*. Senita (*Lophocereus schotii*), saguaro (*Carnegiea gigantea*) and the Mexican cardón (*Pachycereus pringlei*) contain isoquinoline alkaloids

(Meyer & Fogleman 1987). However, there is a common feature between Sonoran and South American systems, resident fly species evolved tolerance to specific host plant alkaloids, that are, otherwise, toxic to other non-resident species.

The study of the transcriptomic responses to different diets in *D. buzzatii* showed that the secondary host imposes stressful conditions during larval growth that are related to *T. terscheckii* alkaloids. The assertion is based on the analysis of the number of DE genes detected in different comparisons and the analysis of functional enrichment. Numbers of DE genes in comparisons involving native *O. sulphurea*, the only phenylethylamine alkaloid-free treatment, (native *O. sulphurea* vs native *T. terscheckii* and native *O. sulphurea* vs *O. sulphurea* + alkaloids), were two orders of magnitude higher than in comparisons involving two alkaloid treatments. Such transcriptional plasticity may help *D. buzzatii* larva to cope with the stressful rearing conditions imposed by the presence of mescaline like alkaloids in *T. terscheckii* rotting pockets (De Panis et al 2016).

The functional enrichment analyses of DE genes showed that the enriched GO terms in the sets of overexpressed genes in alkaloid-rich treatments are mainly related to stress response and detoxification processes. Among genes involved in stress response we detected those involved in the ubiquitin-proteasome pathway which is responsible for a large amount of intracellular proteolysis, including the selective degradation of oxidative damaged proteins. The ubiquitin-proteasome pathway has also been detected in the responses to rearing in alternative fruits in the tephritid fly *Ragholetis pomonella* and in two cactophilic *Drosophila* reared in alternative hosts, *D. mojavensis* and *D. mettleri* (Smith et al. 2013; Hoang et al. 2015; Ragland et al. 2015).

Concerning detox, P450, UGT, GST, Est and Adh gene families are typically induced when an insect is exposed to plant xenobiotics and insecticides (Heidel-Fischer & Vogel

2015). Moreover, some detox genes, such as *Gstd1*, have been shown to be involved in host use and under selection (Matzkin 2008; Gloss et al 2014). As a matter of fact, genes belonging to these families were not only overexpressed in the presence of cardón alkaloids but also expression patterns permit to differentiate between alkaloid free and alkaloid rich treatments, illustrating the sensitivity of the response orchestrated by these genes to cope with diverse environmental challenges (De Panis et al 2016). .

Drosophila koepferae is the complement of *D. buzzatii* in terms of host plant use. Thus, a comparative transcriptomic approach would be helpful to understand the role of host plant shifts and natural chemical stress in ecological specialization. Preliminary analyses suggest that the transcriptome is less sensitive to alternative rearing media and that the main factor affecting transcriptomic plasticity is the cactus hosts and to a lesser extent the presence/absence of alkaloids (De Panis et al unpublished results).

The evolution of cactophily in the *repleta* group suggests that the acquisition of the ability to cope with an array of toxic compounds present in rotting cacti allowed some subgroups to invade and diversify in Neotropical deserts, areas that are generally unfriendly to other *Drosophila* (Wasserman 1982). One of the radiations of the *repleta* group in South America is the *D. buzzatii* cluster. Within the cluster, *D. buzzatii* is the only prickly pear specialist, and, is representative of the plesiomorphic state of host plant use in the *repleta* group (Oliveira et al 2012), since *D. koepferae* and the remaining five species are mainly columnar cactus dwellers (Manfrin & Sene 2006; Hasson et al 2009). Thus, diversification in the *D. buzzatii* cluster certainly involved a history of specialization to columnar cacti and alkaloid tolerance from a more generalist ancestral stock with the ecological characteristics of present day *D. buzzatii*. However, to shed light on the evolutionary history of host plant

use and to establish whether our observations can be extrapolated to other guilds of related species, studies of independent transitions from prickly pear to columnar cacti, that have occurred several times in the *repleta* group (Oliveira et al 2012) will help to understand the relevance of host plant shifts in diversification and the physiological and genetic mechanisms involved.

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Table 1

A. Comparisons across cacti		
Condition	Cactus	
	O. sulphurea	*T. terscheckii*
Native	61	3556
+ Alkaloid	7	15

B. Comparisons across alkaloid conditions within cactus		
Cactus	Condition	
	Native	+ Alkaloids
O. sulphurea	32	1007
T. terscheckii	8	28

C. Comparisons across alkaloid conditions after pooling across native cactus conditions		
	Condition	
	Native	+ Alkaloids
O. sulphurea + T. terscheckii	0	34
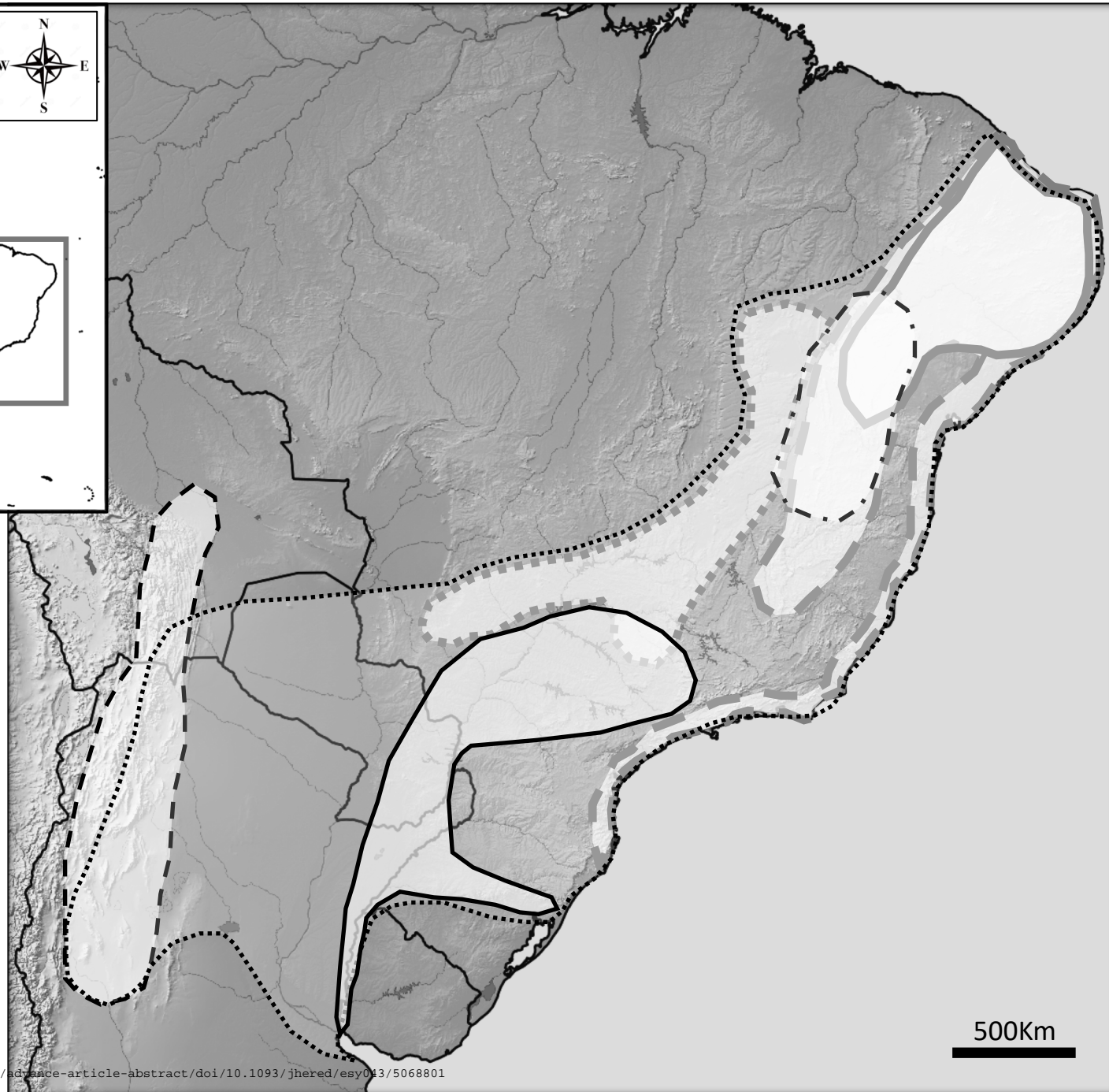
Legend:
- *D. antonietae*
- *D borborema*
- *D. buzzatii*
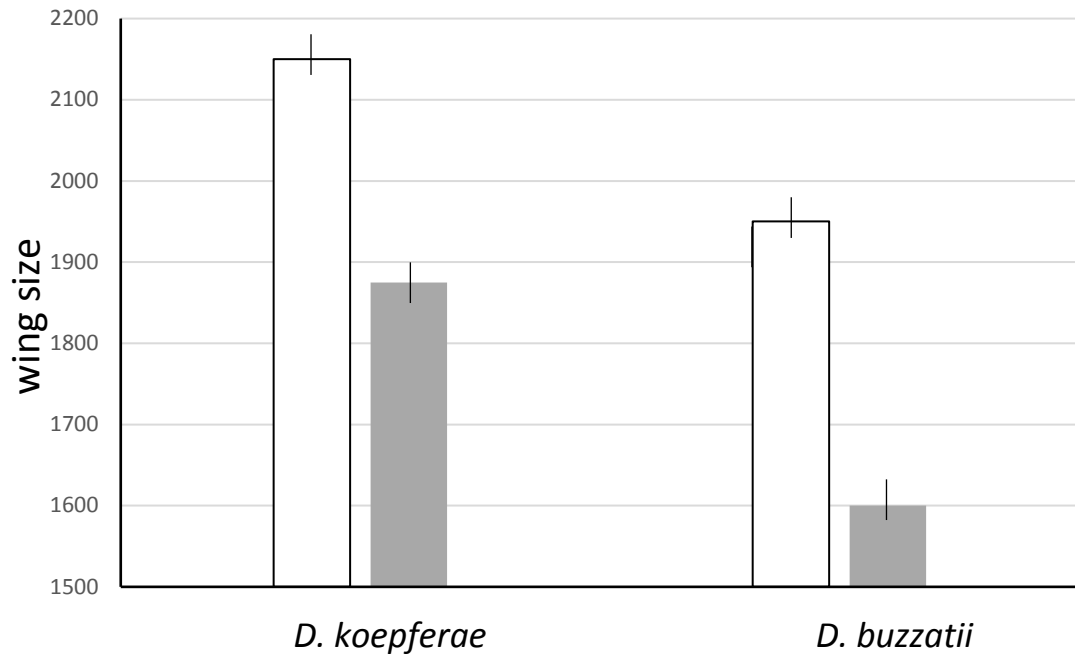- *D. gouveai*
- *D. koepferae*
- *D. serido*
- *D. seriema*

500Km
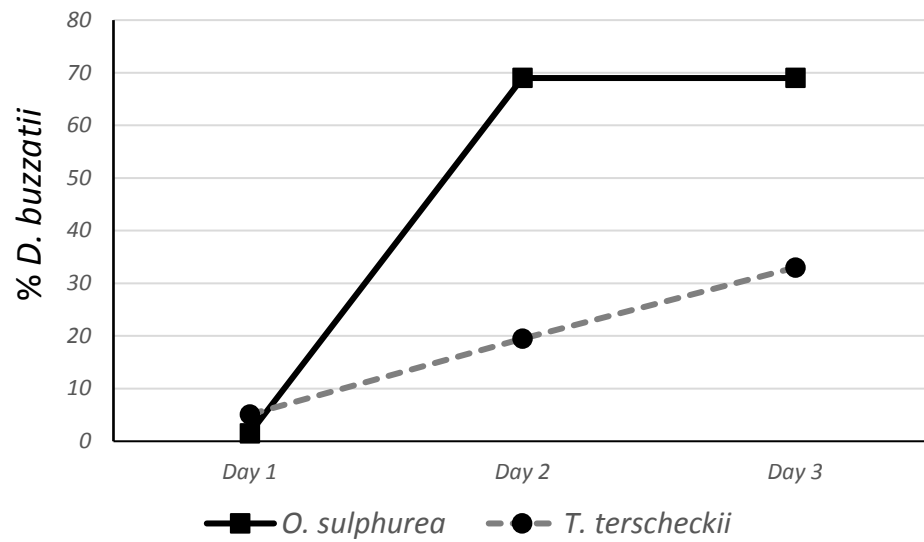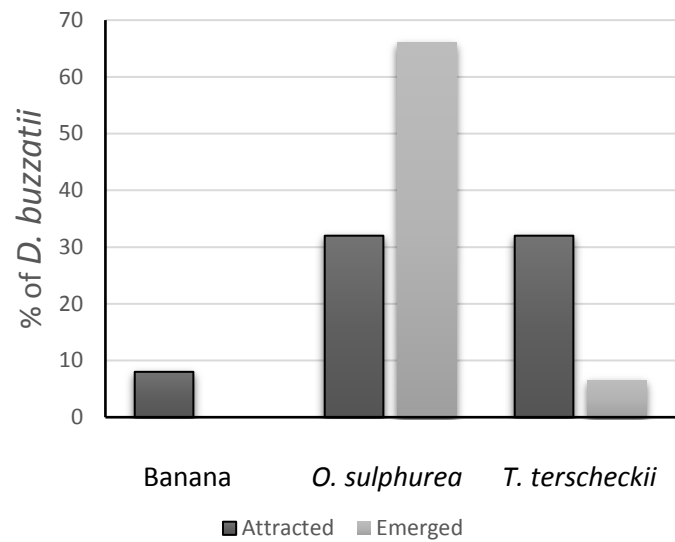
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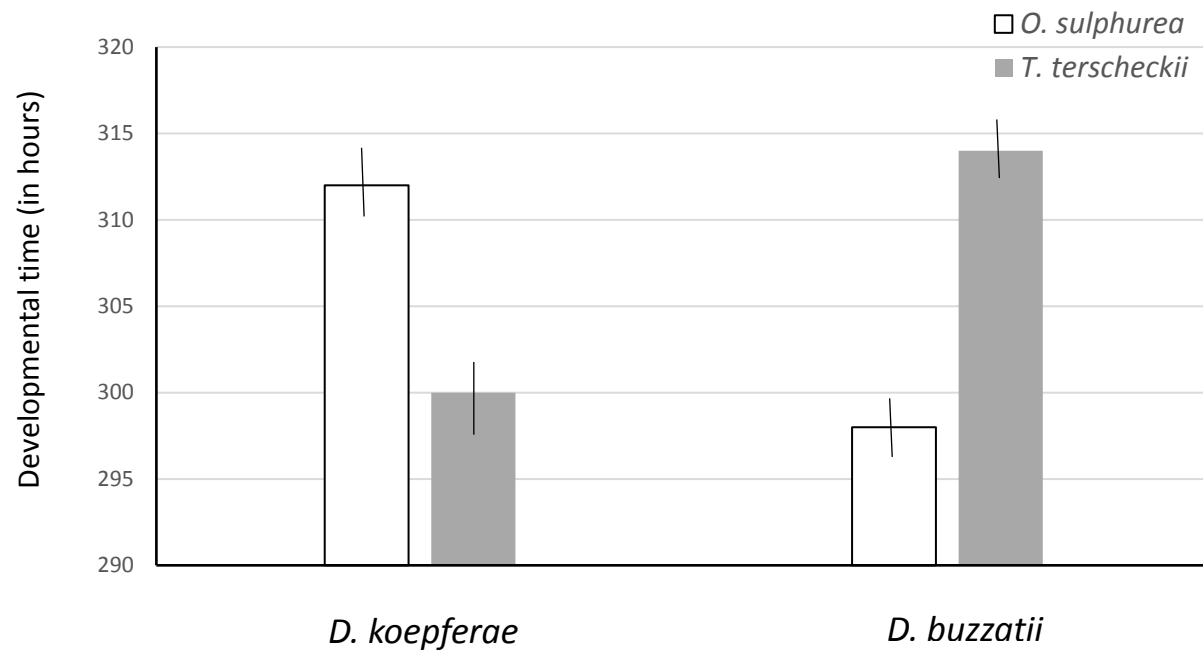
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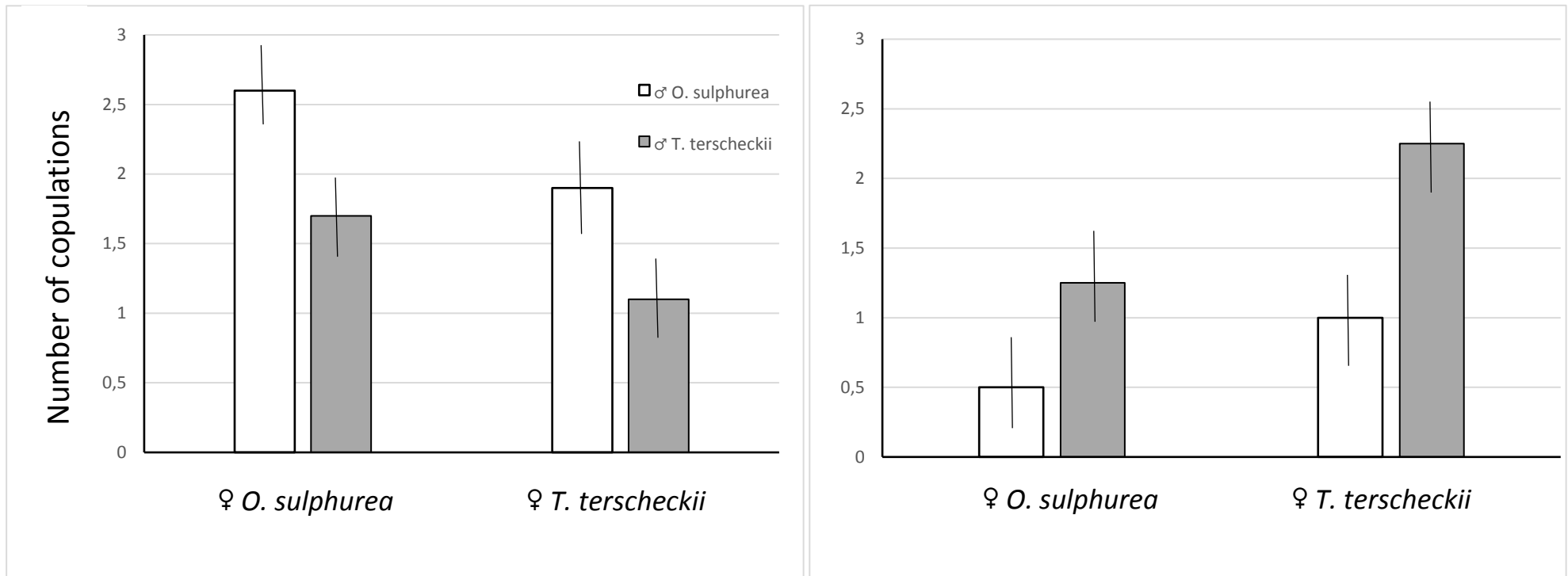
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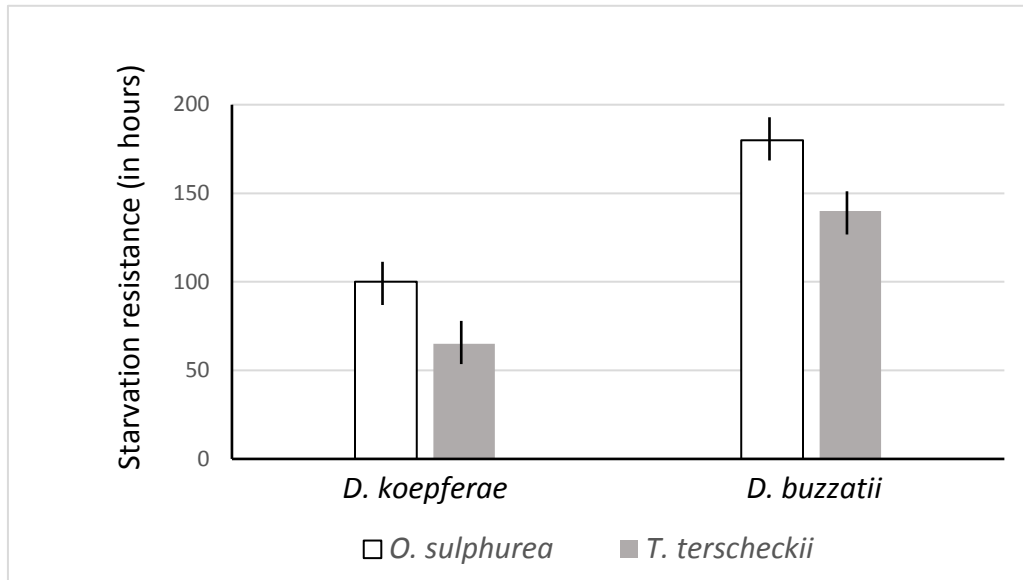
Figure 5
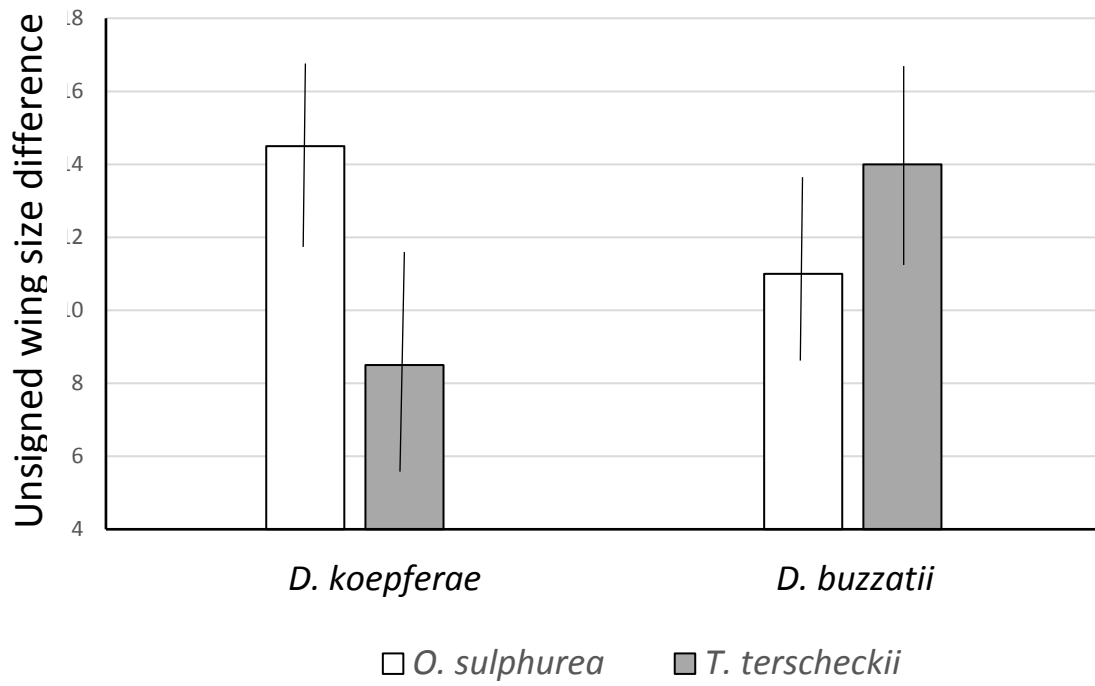
Figure 6

Figure 7

Figure 8