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Cactus–fungi interactions mediate host preference in cactophilic *Drosophila* (Diptera: Drosophilidae)

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The cactophilic flies *Drosophila buzzatii* and *Drosophila koepferae* are generally each associated with a different host cactus, although resource sharing can occur in regions of sympatry. Host choice has been shown to affect several fitness-related traits, but the mechanisms determining it are poorly understood. We investigate how alternative cacti and cactophilic fungi modulate adult host preference (olfaction preference and oviposition behaviour) in both species. All aspects of the flies' resource selection behaviour seem to be driven by both the cactus and the microorganism encountered. In the presence of some fungi, both fly species exhibit strong preferences for their respective primary hosts, while other fungi obliterate differences in preference. Similarly, oviposition behaviour is strongly modulated by particular host–fungus combinations. Overall, the observed patterns of host selection and exploitation in these flies appear to be largely determined by the interaction between the cactus species and only a subset of cactophilic fungi, including the filamentous fungus *Bisifusarium lunatum* and the yeast *Sporopachydermia cereana 'australis'*. The evolution of alternative strategies associated with the election of natural breeding resources has played a crucial role in the divergence of the *D. buzzatii* and *D. koepferae* lineages and might be based on relatively simple decision-making scenarios.

ADDITIONAL KEYWORDS: cactophilic *Drosophila* – cactus – fungi – host choice – olfactory preference – oviposition behaviour.

INTRODUCTION

The family Drosophilidae comprises a huge diversity of saprophytophagous clades whose members rely almost entirely on decaying plant matter as feeding and breeding sites. These flies are involved in complex, long-standing mutualistic interactions with the microbial communities found in their host plants (Ganter, 2006; Starmer & Lachance, 2011). Plant resources provide quality-poor nutrition that is enhanced by the yeasts involved in the decay of plant tissues (Sang, 1978; Begon, 1982). Microbes make nutrients available to the flies by decomposing plant tissues or serving as a direct food source, and themselves benefit by using flies as dispersion vectors (Gonzalez, 2014).

Yeasts (defined as fungi that reproduce primarily by budding or fission, and are therefore single-celled organisms for most of their life cycle; Kurtzmann & Fell, 1998) and bacteria have both been shown to influence most aspects of *Drosophila* biology, including development, gene expression, mating, oviposition, larval feeding choice and food processing (Heed *et al.*, 1976; Anagnostou, Dorsch & Rohlfs, 2010; O'Connor *et al.*, 2014). Insect–microbe interactions are known to contribute to reproductive isolation (Barker, Starmer & Fogleman, 1994; Sharon *et al.*, 2010; Etges & de Oliveira, 2014), host shifts and colonization of new niches (Heed, 1971; O'Grady *et al.*, 2011), as well as increased rates of diversification (O'Connor *et al.*, 2014). This phenomenon is best observed during episodes of evolutionary

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radiation (Janson *et al.*, 2008), as in the case of the *Drosophila repleta* species group (Throckmorton, 1975). This group of flies was able to diversify in arid regions of the Neotropics because of their capacity to breed and feed on cacti (Wasserman, 1982; Durando *et al.*, 2000; Oliveira *et al.*, 2012), a family of plants that underwent a recent radiation (30–35 Mya) concomitant with the increase in atmospheric CO_2 and aridity in the Americas (Arakaki *et al.*, 2011; Majure *et al.*, 2012).

The cactus–yeast–*Drosophila* system has long been held up as a model in evolutionary biology (Barker & Starmer, 1999; Markow & O'Grady, 2008), and there is a wealth of literature regarding its precise dynamics in the Sonoran desert (Heed & Mangan, 1986). The process of cactus tissue decay is initiated when the plant is physically damaged or begins to senesce (Lachance, Starmer & Phaff, 1988; Fogleman & Foster, 1989). Flies attracted to the initial phases of decay inoculate, while feeding, an array of specialized cactophilic fungi that seem to disperse exclusively using these animals as vectors (Ganter, 2011). The microbial community grows vigorously on the decaying tissues, broadly modifying the resource both chemically and physically. During this process, yeasts produce host-specific volatile profiles (used by the flies as cues to find suitable breeding sites), release nutrients, detoxify the medium and serve as a direct food source for the developing progeny larvae (Fogleman & Foster, 1989; Starmer & Aberdeen, 1990; Barker & Starmer, 1999; Fogleman & Danielson, 2001). Several studies have shown that different host plants house differentiated yeast communities even in sympatry (Starmer & Fogleman, 1986; Ganter, 1988, 2011; O'Connor *et al.*, 2014), paralleling the specificity observed in *Drosophila*–host relationships (Heed, 1968; Fogleman & Abril, 1990). Although cactus chemistry plays a central role in the ecological and evolutionary dynamics of these cactophilic *Drosophila* (Fogleman & Heed, 1989; Fogleman & Abril, 1990), the processes of host plant selection and exploitation are very likely shaped by a combination of properties of both cactus hosts and associated microbial communities. However, this three-way interaction between hosts, microbes and insects remains poorly studied (Ganter, 2006; Crowley-Gall, Diefendorf & Rollmann, 2017).

The South American cactus–yeast–*Drosophila* model system offers an opportunity to investigate the roles that host plants and microorganisms play in modulating the complex behaviour of host plant choice (Manfrin & Sene, 2006; Hasson *et al.*, 2009). This system involves the cactophilic species *Drosophila buzzatii* (Patterson & Wheeler) and *Drosophila koepferae*(Fontdevila & Wasserman) (Hasson, Naveira & Fontdevila, 1992). The former uses necrotic cladodes of several *Opuntia* species as primary hosts, while the latter mainly exploits columnar cacti of the genera *Cereus* and *Trichocereus* (Hasson *et al.*, 1992, 2009); however, both species can be recovered from the same rotting pockets in areas of sympatry (Hasson *et al.*, 1992, 2009; Soto *et al.*, 2012). Resource sharing among sympatric species strongly contrasts with the general specificity reported in other clades of *Drosophila*, such as the flies that inhabit the Hawaiian archipelago or the guild of cactophilic species living in the desert of Sonora (Fogleman & Abril, 1990; Ort *et al.*, 2012). Previous studies showed that insect performance, measured in terms of several fitness-related traits, depends on the breeding resource (Fanara, Fontdevila & Hasson, 1999; Soto *et al.*, 2008a, b, 2012). *Drosophila buzzatii* is more viable, develops faster, attains a larger adult body size and is more resistant to starvation when raised on the decaying cladodes of *Opuntia sulphurea* (G. Don in Loudon) than on the columnar *Trichocereus terscheckii* [(Parm. ex Pfeiff.) Britton & Rose]. *Drosophila koepferae*, on the other hand, performs better when raised on decaying *T. terscheckii*, although its response to different cactus rearing media is not as consistent as in its sister species (Carreira *et al.*, 2006; Soto *et al.*, 2008a, b; Hasson *et al.*, 2009; Soto *et al.*, 2012). The variation in performance has been attributed to differences in the chemistry of both resources (Corio *et al.*, 2013; Padró & Soto, 2013; Carreira *et al.*, 2014; Soto *et al.*, 2014).

From an evolutionary perspective, natural selection should favour females able to discriminate between hosts and who prefer the most appropriate option for their progeny, especially when host plants differ in their suitability and immature stages are confined to the host selected by their mother (Thompson, 1988; Yang *et al.*, 2008; Soto *et al.*, 2012). In this context, host preference is defined as the consistent use or choice by individuals of a host among several alternatives (Schoonhoven, van Loon & Dicke, 2005) and is a central issue in the study of the evolutionary history of phytophagous insects (Gripenberg *et al.*, 2010). The existence of regions where individuals of both *D. buzzatii* and *D. koepferae* systematically choose secondary hosts, resulting in less fit adults, seems at first an evolutionary paradox. However, this conundrum may be resolved by incorporating the microbial community into the study of host plant choice, and the assessment of the interaction between all elements of the system. A recent survey of the microbial species recovered from rotting pockets of the main hosts of cactophilic *Drosophila* showed that the microbiota of *T. terscheckii* is a subset of that found in the prickly pear *O. sulphurea* (Mongiardino Koch *et al.*, 2015), instead of being highly differentiated as in other cactus–yeast–*Drosophila* systems (Starmer & Fogleman, 1986; Ganter, 1988; Crowley-Gall *et al.*, 2017). Thus, it is possible that this peculiar nested pattern may be the key to understanding what drives attraction and overall preference of *D. buzzatii* and *D. koepferae* for their hosts.

The aim of the present study is to investigate whether cactus–fungi interactions modulate adult

host preference in the pair of cactophilic sister species *D. buzzatii* and *D. koepferae*. In particular, we hypothesize that *Drosophila* species, although primarily attracted to their respective primary hosts, also accept alternative hosts depending on the fungal species fermenting the resource.

MATERIAL AND METHODS

Collection of material, stock maintenance and preparation of experimental media

Fly collections were carried out in March 2014, in the Valle Fértil Natural Reserve (30°41′26.5″S 67°29′45.5″W, San Juan Province, Argentina), a place where *D. buzzatii* and *D. koepferae* coexist and *O. sulphurea* and *T. terscheckii* are, respectively, their only hosts. Flies were collected by net sweeping on yeast-banana baits, sexed upon arrival to the laboratory and used to generate isofemale lines of both *Drosophila* species (Hoffmann & Parsons, 1988). With the aim to maximize genetic variability, two outbred stocks, one of each species, were founded using 20 males and 20 females of each one of seven randomly chosen isofemale lines. These stocks were reared and fed in bottles with standard laboratory instant medium (Carolina Biological Supply Company) under identical laboratory conditions for two generations before the onset of the experiments.

Cactophilic fungal strains were collected in the same region by aseptically sampling necrotic wounds of *O. sulphurea* and *T. terscheckii*. A standard protocol was used for the isolation and purification of fungal isolates from cactus rotting pockets (details in Mongiardino Koch *et al.*, 2015). Species identification of fungal isolates was accomplished using a combination of morphological, physiological and molecular approaches. Briefly, the D1/D2 domain of the large subunit 26S rDNA gene was amplified and compared with reference sequences using the basic local alignment search tool of the BLAST software program from NCBI (BLAST, 2011). In addition, the API C aux system (Biomerieux) was used to physiologically characterize fungal isolates, and the physiological profiles were compared to those reported in the literature. For this study, we used strains of the four most abundant species of cactophilic fungi found in the area: *Bisifusarium lunatum* [(Ellis & Everh.) L. Lombard and Crous], *Pichia cactophila* (Starmer, Phaff, M. Miranda and M.W. Mill), *Dipodascus australiensis* (von Arx & J.S.F. Barker) and *Sporopachydermia cereana 'australis'* (Rodr. Mir). These species were detected in more than 95% of the samples, representing ~75% of the total cactophilic isolated strains. *Bisifusarium lunatum* and *P. cactophila* were found in both cactus hosts, while *Di. australiensis* and *S. cereana 'australis'* were only found in *O. sulphurea* necroses (Mongiardino Koch *et al.*, 2015).

We also collected fresh tissues of *O. sulphurea* and *T. terscheckii*. Pieces of fresh cacti were stored at −25 °C until use in the preparation of two types of 'semi-natural' media, each containing tissues of only one cactus species. For this purpose, the cactus tissues were ground in a blender and sterilized in an autoclave to control the microbial communities present in the samples. After cooling, 3 g of cactus tissue were placed in individual glass vials (see Soto *et al.*, 2012 for details). The vials were inoculated with 1 mL of a suspension containing 10^7 cells of one of the four fungus species, obtaining a final cell density per milligram of cactus tissue within the range observed in natural rots (Mongiardino Koch *et al.*, 2015). Suspensions were obtained by restreaking fungal strains on glucose–peptone–yeast extract agar, incubating for 48 h at 27 °C, suspending colonies in sterile saline solution and adjusting cell concentration by cell counting in a Neubauer chamber. After inoculation, vials were incubated at 25 °C for 48 h before the onset of experiments.

Behavioural assessment: measuring the effect of yeasts and cactus on adult host preference

The objective of the experiments described below was to investigate whether the preference of adult flies for two different hosts is conditioned by the presence of specific fungal species. To this end, we evaluated adult olfaction preference and oviposition behaviour using two types of preference assays.

First, as control assays for both behavioural traits, we offered fresh cactus vs. distilled water to the flies in the Olfaction preference studies and fresh cactus vs. agar-agar for the Oviposition preference assay. For both traits, 15 replicates were performed for each combination of cactus and *Drosophila* species.

Olfaction preference

Olfaction preference was assessed using a 'Y-shaped tube' olfactometer (Fuyama, 1976). Ten sexually mature females (4–5 days old) were released at the base of the tube. At each opposite end of the Y-shaped tube, 1 mL of tissue from each of the two cactus hosts (*O. sulphurea* and *T. terscheckii*), both inoculated with the same fungus species (i.e. either *B. lunatum*, *P. cactophila*, *S. cereana 'australis'* or *Di. australiensis*), was presented to the flies. Air flow was kept constant with the use of an air pump. After 15 min, we registered the number of flies in each arm of the tube. Flies that remained at the base of the Y-shaped tube after 15 min of the initiation of the assay were not considered in subsequent statistical analyses. We constructed a choice index as the number of adults attracted to *O. sulphurea* divided by the

total number of flies in both arms of the Y-shaped tube. Ten replicates were run for each combination of fungus and *Drosophila* species.

Oviposition behaviour

For oviposition behaviour assays, 20 pairs of sexually mature flies (4–5 days old) were released in chambers with four plates containing agar-agar and a homogenous layer of fermented cactus tissue spread on top. Two of the plates contained *O. sulphurea* and the other two contained *T. terscheckii*; all four plates in each replicate were inoculated with only one fungus species. After 24 h, all plates were removed and photographed with a digital camera attached to a binocular microscope for egg counting. Twenty replicated chambers were run for each combination of fungus and *Drosophila* species.

To evaluate oviposition behaviour, we counted the total number of eggs on each plate using the program TPSDIG v.1.4 (Rohlf, 2001).

DATA ANALYSIS

Statistical analyses were performed in the R environment (R Core Team, 2015) with packages MASS (Venables & Ripley, 2002), multcomp (Hothorn *et al.*, 2008) and car (Fox & Weisberg, 2011).

A generalized linear model (GLM) with binomial distribution was applied for the olfaction preference analysis. Strong overdispersion was detected in the analysis of the number of eggs, so SE were corrected using a negative binomial GLM. For both analyses, the meaningful interaction terms were selected using a likelihood ratio test. The final model included single effect factors and described only the interaction between *Drosophila* and yeast species.

Both analyses included two explanatory variables: *Drosophila* species (*D. koepferae* and *D. buzzatii*) and yeast species (*B. lunatum*, *S. cereana 'australis'*, *P. cactophila* and *Di. australiensis*). In oviposition assays, we also included cactus species (*O. sulphurea* and *T. terscheckii*) as a factor.

In the case of olfaction preference, we tested for significant deviations from random behaviour by performing 10^5 coin-flipping simulations, each of which consisted of ten replicates, as in the olfaction experiments. A two-way ANOVA revealed no significant effect of *Drosophila* or fungal species, nor of an interaction between the two, on the number of flies that moved from the base of the tube to a resource (all $P > 0.18$). Furthermore, the distribution of the number of flies that reacted to the stimuli did not deviate significantly from a normal distribution (Shapiro-Wilk test, $W = 0.97$, $P = 0.06$). Therefore, parametric bootstrapping was performed in each replicate to select a number of flies from a normal distribution with a mean and SD as observed in the assays ($\mu = 5.11$, SD = 2.42; the distribution was truncated so as to generate values between 0 and 10). Each fly then selected a host at random, and the same choice index was calculated and averaged across all replicates for a given simulation. The results were used to build a 95% confidence interval (CI) for the olfaction preference index.

RESULTS

Olfaction preference

To evaluate these data obtained in the control assay, we performed a permutation test (10 000 permutations, $\alpha = 0.025$) to determine whether flies were choosing randomly between the alternatives offered. Our results showed that both *Drosophila* species preferred cacti over distilled water, independent of the cactus species offered. *Drosophila buzzatii* had a choice index of 0.68 for *O. sulphurea* ($P < 0.025$) and 0.73 for *T. terscheckii* ($P < 0.001$), while *D. koepferae* had an index of 0.69 for *O. sulphurea* ($P < 0.025$) and 0.74 for *T. terscheckii* ($P < 0.01$). Fewer than 30% of flies of either species showed no preference (remained at the base of the Y-shaped tube).

Drosophila buzzatii and *D. koepferae* exhibited different behaviours, and also showed responses that were strongly dependent on the fungus species offered in cactus choice experiments (Fig. 1). Despite the

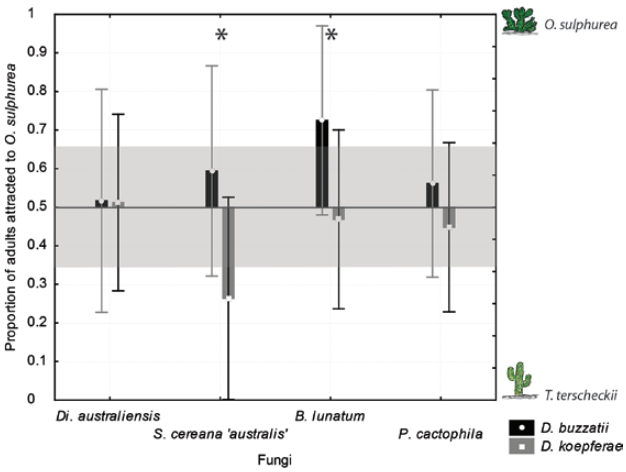

Figure 1. Mean and SD of the proportion of adults of each *Drosophila* species attracted to *Opuntia sulphurea* depending on the fungi present (values close to 1 represent olfaction preference for *O. sulphurea* and values close to 0 represent preference for *Trichocereus terscheckii*). Asterisks (*) denote significant differences between *Drosophila* species, and the grey area represents a 95% CI around 0.5 (i.e. random host selection).

markedly different patterns observed in the profile plots, the *Drosophila* × Fungus interaction proved to be non-significant (Table 1A). Because the results bordered statistical significance, however, we further investigated this interaction to rule out the test's lack of power as a potential explanation. Simple effect analyses in experiments involving each of the fungal species showed that differences between *D. buzzatii* and *D. koepferae* in adult olfaction preference were significant in the assays involving *B. lunatum* ($\chi^2 = 5.107$, d.f. = 1, $P < 0.05$) and *S. cereana 'australis'* ($\chi^2 = 11.897$, d.f. = 1, $P < 0.01$). It was only in the assays involving one of these two fungi that fly behaviour differed from random choice (Fig. 1). *Drosophila buzzatii* preferred its primary host in the presence of *B. lunatum*, as did *D. koepferae* in the presence of *S. cereana 'australis'*. In both cases, the preference of the other fly species was not beyond the 95% CI. Behavioural differences between *Drosophila* species did not deviate from random expectations in the assays involving either *P. cactophila* or *Di. australiensis*.

Oviposition behaviour

The control assay showed that both *Drosophila* species prefer to place their eggs on cacti rather than on agar-agar, independent of the cactus species offered. Specifically, *D. buzzatii* laid 93–99% of its eggs on cactus ($\chi^2_{O.\ sulphurea\ \text{vs. Agar-Agar}} = 32.218$, d.f. = 1, $P < 0.001$; $\chi^2_{T.\ terscheckii\ \text{vs. Agar-Agar}} = 4.557$, d.f. = 1, $P < 0.05$), and *D. koepferae* laid 100% of its eggs on cactus ($\chi^2_{O.\ sulphurea\ \text{vs. Agar-Agar}} = 49.884$, d.f. = 1, $P < 0.001$; $\chi^2_{T.\ terscheckii\ \text{vs. Agar-Agar}} = 246.12$, d.f. = 1, $P < 0.001$).

Table 1. Analysis of deviance with a GLM with (A) binomial distribution for the adult olfaction behaviour and (B) negative binomial distribution for number of eggs laid in relation to cacti (*Opuntia sulphurea* and *Trichocereus terscheckii*) inoculated with four different fungi (see text for explanation)

	χ^2	d.f.	*P*
(A) Olfaction preference			
Drosophila	11.259	1	< 0.001
Fungus	4.021	3	0.259
Drosophila × Fungus	7.102	3	0.068
(B) Oviposition preference (number of eggs)			
Drosophila	35.771	1	< 0.001
Fungus	30.708	3	< 0.001
Cactus	133.717	1	< 0.001
Drosophila × Fungus	10. 637	3	< 0.05

Data analysis of oviposition behaviour assays in different cactus media inoculated with one of the evaluated fungal species revealed that the *Drosophila* × Fungus interaction was significant and that both *Drosophila* species laid more eggs in *O. sulphurea* than in *T. terscheckii* (Table 1B; Fig. 2). *A posteriori* pairwise Tukey comparisons showed that differences between fungi were only significant in *D. buzzatii* (Fig. 2). The number of eggs laid by *D. buzzatii* in substrates inoculated with *S. cereana 'australis'* was higher than in substrates inoculated with *Di. australiensis* or *B. lunatum* (*z* value = 5.219, $P < 0.001$ and *z* value = 4.227, $P < 0.001$, respectively). In addition, we observed that *D. buzzatii* consistently laid more eggs than *D. koepferae*, evidencing a greater fecundity (Fig. 2). This difference can mostly be accounted for by the fact that *D. buzzatii* laid more eggs than *D. koepferae* in the

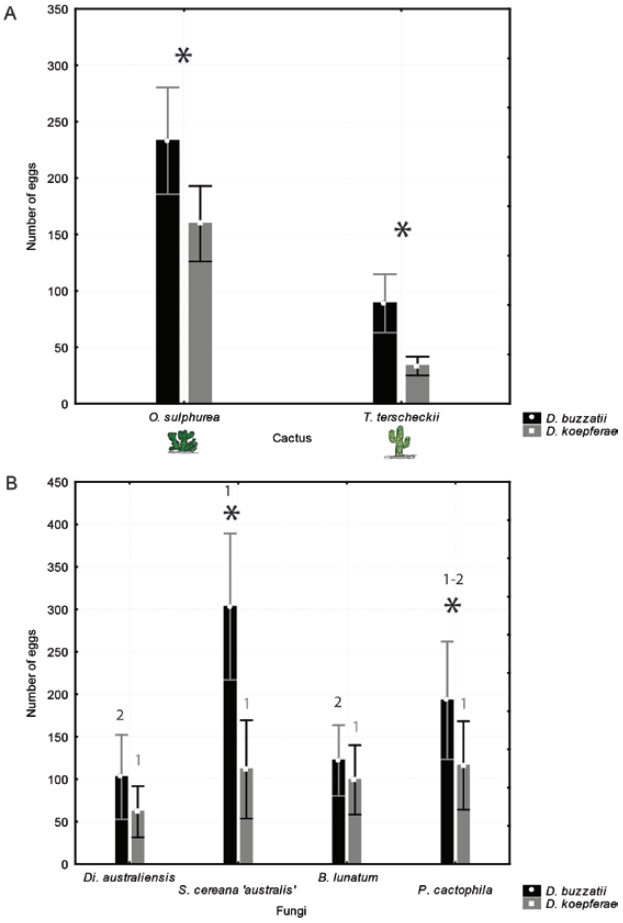

Figure 2. Mean and SD of the number of eggs laid depending on (A) cactus host and (B) the fungal species, for each *Drosophila* species. Asterisks (*) denote significant differences between *Drosophila* species. Results of *a posteriori* Tukey's comparisons are represented with black numbers for *Drosophila buzzatii* and grey numbers for *Drosophila koepferae*.

presence of both *S. cereana 'australis'* and *P. cactophila* (z value = −5.280, $P < 0.001$ and z value = −3.138, $P < 0.05$, respectively).

DISCUSSION

Our study highlights the importance of including the microbial community in preference assays. In fact, our results not only reveal the roles played by different microorganisms as possible environmental cues for flies to select specific hosts among alternatives but also demonstrate that the flies' response to these cues is species-specific. Surprisingly, the olfaction preference assay revealed that neither *D. buzzatii* nor *D. koepferae* are invariantly attracted to their primary hosts but rather that their behaviour is dependent on the cactophilic fungi present in cactus necrosis. Preference for alternative hosts differed between species only when either *B. lunatum* or *S. cereana 'australis'* were added to the cactus media. *Drosophila buzzatii* showed a strong preference for *O. sulphurea* when both cacti were fermented by *B. lunatum*, while *D. koepferae* was significantly attracted to *T. terscheckii* only in the assays involving *S. cereana 'australis'*. The latter is an unexpected result since *S. cereana 'australis'* has never been isolated from necrotic *T. terscheckii* in the field (see below). Nonetheless, these results indicate that the same volatile stimulus elicited species-specific responses in this pair of sibling cactophilic flies, modulating behaviour towards alternative resources. Our results also show that *P. cactophila* and *Di. australiensis* did not affect attraction. This does not imply that these yeasts do not influence olfaction behaviours in *D. buzzatii* and *D. koepferae*, but rather indicates that the olfaction stimuli generated by these microorganisms do not result in differential attraction to alternative hosts. However, we did not test whether or not they could have an effect on the flies' attraction to alternative hosts when present in combination with other cactophilic yeasts.

The analysis of oviposition behaviour data showed that females of both species consistently laid more eggs in *O. sulphurea* than in *T. terscheckii*, regardless of the yeast present. This result is not surprising for *D. buzzatii* since prickly pears are its preferred host (Fanara *et al.*, 1999; Soto *et al.*, 2012); however, it is an unexpected outcome for *D. koepferae*, a columnar cactus specialist. Regarding the effect of microorganisms on oviposition behaviours and fecundity, we observed that egg-laying patterns differed sharply between species. *Drosophila koepferae* laid similar numbers of eggs irrespective of the species of microorganism, whereas *D. buzzatii* females exhibited a more plastic behaviour, with the number of eggs laid depending on the microorganism encountered. In particular, *S. cereana 'australis'* seemed to be the best stimulus for *D. buzzatii* since females laid significantly more eggs in assays in which it was present than in those fostering other microorganisms. Differences in fecundity between *D. buzzatii* and *D. koepferae* have already been described (Fanara & Hasson, 2001). However, this is the first report showing that fecundity is differentially modulated by the microorganism present in the cactus medium offered to *D. buzzatii*, as well as revealing that this pattern is not seen in its sibling species *D. koepferae*. Overall, our results suggest that the primary stimulus affecting oviposition behaviour varies between species. In effect, egg-laying behaviour depended on the particular combination of cactus and yeast in *D. buzzatii*, whereas *D. koepferae* was more responsive to the cactus host than to the microorganism or the cactus–microorganism combination.

Traditionally, only yeasts and yeast-like fungi were considered to be native inhabitants of the cactophilic niche, whereas filamentous fungi such as *B. lunatum* were thought to neither exploit this resource nor use flies as vectors (Starmer, Fogleman & Lachance, 1991; Coluccio *et al.*, 2008; Ganter, 2011). However, *B. lunatum* is not only present and extremely abundant in some cactus-dominated regions (Mongiardino Koch *et al.*, 2015) but has also been shown to be vectored by *Drosophila* flies (Swart & Swart, 2003). Different strains of *B. lunatum* (formerly *Fusarium lunatum*; Lombard *et al.*, 2015) have been shown to ferment a variety of sugars, as well as cellulose, directly into ethanol (Ueng & Gong, 1982; Christakopoulos, Macris & Kekos, 1989), which is the main volatile *Drosophila* attractant (Gelfand & McDonald, 1980). In addition, *B. lunatum* has been shown to have an extremely aggressive form of tissue penetration (Flores-Flores *et al.*, 2013), which is likely to impact the rate of tissue liquefaction responsible for the generation of suitable conditions for cactophilic *Drosophila* larvae (Fogleman & Danielson, 2001). One of the most divergent attraction patterns between *D. buzzatii* and *D. koepferae* was observed in the assays in which *O. sulphurea* and *T. terscheckii* were inoculated with *B. lunatum*, suggesting that this species is a generator of important cues that affect resource selection in these flies. Given the abundance of *B. lunatum* in cactus necroses (Mongiardino Koch *et al.*, 2015), its impact in the dynamics of the system and its strong and versatile fermenting capabilities, we hypothesize that the ability to respond to the host-specific pattern of volatiles produced in rotting *Opuntia* cladodes by this filamentous fungus improves the ability of *D. buzzatii* to localize suitable breeding sites.

The other divergent attraction pattern was detected when flies were offered cacti fermented with *S. cereana*

'australis', which is among the most common cactophilic yeasts both locally and globally (Ganter, 2011; Mongiardino Koch *et al.*, 2015). The strong effect elicited by *S. cereana 'australis'* on *D. koepferae* attraction behaviour is very interesting since this yeast has been regarded as an *Opuntia* specialist. Even though it was not isolated from columnar cactus necroses in a recent survey in western Argentina (Mongiardino Koch *et al.*, 2015), there is evidence that *S. cereana 'australis'* can grow in *T. terscheckii* since media prepared with this cactus inoculated with *S. cereana 'australis'* showed an increase in turbidity and a strong alcoholic smell (N. Mongiardino Koch, pers. observ.). There are several explanations that may account for these results, including (1) *S. cereana 'australis'* is excluded from certain stages of decay of *T. terscheckii* by competition with other yeasts, a common interaction among cactophilic microorganisms (Lachance *et al.*, 1988; Ganter & Starmer, 1992) and/or (2) the yeast has not yet been detected due to insufficient sampling. Interestingly, the presence of *S. cereana 'australis'* makes columnar cacti very attractive to *D. koepferae* and has a substantial effect on the oviposition behaviour of *D. buzzatii* when inoculated in *O. sulphurea*. Taken together, these results suggest that the presence of the same yeast species in the necroses of two different cacti has differential effects on host preference and oviposition behaviour in these fly species.

In general, our results show that *D. buzzatii* and *D. koepferae* are capable of a surprising behavioural diversity that ranges from strong preferences for their primary hosts to no preference at all, as well as changes in oviposition behaviour dependent on the microorganisms present in the cactus necroses. These patterns are in line with recent data for the fruit fly *D. melanogaster* showing that volatiles produced by yeasts growing on artificial minimum media induced the same fly behaviour as volatiles produced by yeast fermenting fruits (Becher *et al.*, 2012).

Host shifts have been implicated in the diversification of cactophilic *Drosophila* (Oliveira *et al.*, 2012), as well as in other phytophagous insects (Futuyma & Agrawal, 2009). In this context, our study increases the understanding of the important role that microorganisms play in the cactus–*Drosophila* system since host shifts may simply involve the evolution of differential responses to the volatiles produced by a relatively small number of microbes. Through time, such differential attraction to host plants fermented by different (or even the same) microorganism, along with the dispersal of cactophilic microbes vectored by flies, might result in the establishment of novel three-way interactions by means of the process of niche construction (Odling-Smee, Laland & Feldman, 2003).

In summary, our study shows that olfaction preference and oviposition behaviour are the outcomes of complex interactions among cactus hosts, microorganisms and flies, suggesting a long intertwined evolutionary history. Furthermore, two of the major components of the cactophilic microbiota, the filamentous fungus *B. lunatum* and the yeast *S. cereana 'australis'*, are among the main factors determining patterns of host selection and exploitation by cactophilic *Drosophila*. These microorganisms seem to produce olfactory cues that differentially affect host selection and oviposition behaviour in *D. buzzatii* and *D. koepferae*, suggesting that alternative strategies associated with the election of natural resources evolved after the separation of these lineages from their last common ancestor.

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