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Drosophila pupation behavior in the wild

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Abstract We investigated pupa distributions of *D. simulans*, *D. buzzatii*, *D. melanogaster*, *D. immigrans* and *D. hydei* on a number of natural breeding sites. Pupae of all five species showed aggregated distributions, which prompted us to examine these aggregations in a more detail for two species that commonly co-occur in breeding sites, *D. simulans* and *D. buzzatii*. We found that pupae of both species tend to be aggregated in conspecific clusters. Subsequent experiments revealed that both species are attracted to the odors of other larvae, though only *D. buzzatii* differentiated between conspecifics and heterospecifics (they preferred conspecific). Furthermore, third instar larvae of both species preferred more alkaline substrates. Altogether, our results demonstrate that *Drosophila* species form conspecific pupa aggregations in natural breeding sites, and that pupation site selection depends on interactions among conspecific and heterospecific larvae and on chemical characteristics of the breeding sites.

This paper is dedicated to Professor Kevin Connolly.

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Introduction

In *Drosophila*, selection of pupation site is important to the successful emergency of the adults. *Drosophila* pupation behavior is also related with habitat selection, colonization of new niches and the expansion of populations (de Souza et al. 1970). Thus, pupation behavior of *Drosophila* is one component of the complex of behaviors of the larva that affect the genetic structure of populations (de Bono and Sokolowski 2007). On the other hand, investigations focused on selection of pupation sites in the wild may provide valuable information on evolutionary forces that contribute to local guilds that share common resources coexist and persist through time (Martin and Martin 2001). More specifically, *Drosophila* breeding site characteristics provide a spatial context for understanding the behavior of *Drosophila* larvae in relationships with population biology and community ecology. Cohabitation of larval resources by multiple species could, for example, drive larvae of each species to pupate in different microhabitats. Thus, the behaviors of *Drosophila* larvae involved in pupation site selection are related with population size, structure and assembly of *Drosophila* species communities. While hypothesis on habitat selection behavior by adults have been tested (Mery and Kawecki 2004), we know very little on genetic, ecological, and evolutionary consequences of pupation site selection made by larvae.

Pupation behavior in the wild implies to distinguish between conspecific and heterospecific larvae that live in the same breeding site unit, and to respond to physical features of the breeding site (Medina-Muñoz and Godoy-Herrera 2004).

Thus, pupation behavior is important for fitness because considers habitat use and interactions between biotic and abiotic influences, and this has implications for coexistence of "scramble" type competitor species that share common resources (Martin and Martin 2001). On the other hand, pupa aggregations of *Drosophila* observed in the wild provide wide opportunities to be infected by, for example, parasitoids wasps (Pannebakker et al. 2008). Consequently, investigations on the behavior of *Drosophila* larvae in natural rearing environments are central to our understanding of population genetics and evolution of the genus (Carson 1971).

There are demonstrations of the influence of the genotype on aspects of pupation behavior (review in Sisodia and Singh 2005). For example, pupation height in shell vials in *Drosophila ananassae* is under polygenic control and most of the variance is additive (Singh and Pandey 1993). Pupation by *D. melanogaster* outside the food cup is dominant over pupation inside the cup; there is also additive variation (Godoy-Herrera et al. 1989). Features of pupation site selected by larvae also affect pupal survival of *D. melanogaster* (Rodriguez et al. 1992). Larvae of *Drosophila melanogaster*, *Drosophila pavani* and *Drosophila gaucha*, and of the reciprocal inter-specific hybrids between the latter two species react to humidity, light and to substrate texture and consistency when they search for pupation sites (Wong et al. 1985; Godoy-Herrera et al. 1989; Godoy-Herrera and Silva-Cuadra 1997, 1998).

In the wild, species of *Drosophila* use a variety of breeding sites (Powell 1997), and this is reflected in selection of pupation site. Thus, *D. melanogaster* larvae prefer to pupate outside the breeding site, while *D. simulans* pupate on the fruit (Vandal et al. 2008). How

Drosophila larvae select pupation sites in the wild is poorly understood (Medina-Muñoz and Godoy-Herrera 2004). It is therefore important to ask whether *Drosophila* larvae respond to stressful conditions imposed by fermentation processes within breeding sites and to the presence of other *Drosophila* larvae, and how these behaviors contribute to pupation site selection. This type of research should shed light on the manner by which larvae of different *Drosophila* species partition resources in shared environments (Medina-Muñoz and Godoy-Herrera 2004; Mery and Kawecki 2004).

Most studies on larval pupation behavior of *Drosophila* have used food vials as the substrate. Pupation site preference has been measured by the distance between the surface of the substrate and the pupa location (review in Sisodia and Singh 2005). This experimental design provides a limited insight into features of natural breeding site that regulate choice of pupation site. Here we report on larval pupation behavior of some *Drosophila* species in relationship with ecology of their breeding sites. In this study, we analyze pupation site preferences for several *Drosophila* species (*Drosophila simulans*, *D. melanogaster*, *Drosophila immigrans*, *Drosophila hydei,* and *D. buzzatii*) by examining pupa positions in naturally occurring breeding substrates.

Because we found that pupae of each of these five species were aggregated in the substrates, we then performed a set of experiments aimed at identifying cues involved in aggregation behavior. Specifically, we investigated the response of *D. simulans* and *D. buzzatii* third instar larvae to conspecific and heterospecific odors, and to acidity/alkalinity of breeding substrates (David et al. 1983). These experiments suggest that pupation site selection behavior in *D. simulans* and *D. buzzatii* is influenced by the presence of other larvae and by chemical characteristics of breeding sites.

Materials and methods

Breeding site collections

We collected 757 fermented fruits of apple (Red Delicious variety), red grape (País variety), pear (Abbot Fedel variety), apricot (Imperial variety), pumpkin (Camote variety), paprika (Capitrano variety) and medlar (Tanaka variety) in Olmué, 120 km northwest of Santiago. In Til-Til, 50 km away from Santiago, we collected 34 pieces of necrotic cactus tissue, *Echinopsis chilensis*, and 33 decaying fruits and 18 pieces of necrotic tissue (cladodes) of prickly pear, *O. ficus-indica*. We used a random number table to randomly select plants for collections. The collections were made in April of 2006 (Autumn in Chile) when Chilean populations of *Drosophila* reach their peak of abundance (Brncic et al. 1985).

"Nearest neighbor" analysis in the wild

Between 55.32 and 76.71% of *D. melanogaster*, *D. simulans*, *D. buzzatii*, *D. immigrans* and *D. hydei* pupae were on the fruit skin, while 44.68–23.29% were found underneath the skin. Pupae found on the ground were excluded from the analysis. We recorded the position of each pupa on the substrate, and measured the distance to the nearest neighbor. We then individually deposited each pupa in a vial containing a piece of moistened filter paper, and stored them in an incubator at 24°C until adults emerged and were identified to species.

Pupa distributions of each one of the five *Drosophila* species (Table 1) were analyzed using the "nearest neighbor" method of Clark and Evans (1954). First, we estimated the average distance to the nearest neighbor (rA) by using a 0.5 cm Cartesian grid, and then compared this with the expected value (rE) for the same number of individuals randomly distributed on an area of equal size ($rE = 1/2\sqrt{\rho}$), where ρ is the pupa' density. The ratio $R = rA/rE$, reflects the form of the spatial distribution of the individuals (aggregated, random, overdispersed) with values ranging between $R = 0.0$ (maximum aggregation) and $R = 2.15$ (uniform). When individuals are randomly distributed, $R = 1.0$.

We also used the distance between pupa of the observed sample and its nearest neighbor to calculate *G* function for the five *Drosophila* species (Diggle 1983).This function estimates whether observed samples have a regular, random or aggregated pattern. *G* functions were compared with the expected theoretical function obtained under the null hypothesis of complete spatial randomness, by applying a test statistic in which the expected null hypothesis value is zero at all distances (details in Diggle 1983). When the null hypothesis is rejected, the sign of the difference between the observed and expected theoretical distributions indicates whether there is a tendency towards aggregation or regularity, with positive values indicating aggregation, and negative values regularity (Diggle 1983). Significance of the G function values were tested using the Montecarlo procedure (Diggle et al. 1991).

To test whether selection of pupation sites by *D. buzzatii* and *D. simulans* is affected by the presence of heterospecific larvae, we analyzed single and mixed pupal aggregations of these two species, on decaying fruits and necrotic tissues (cladodes) of prickly pear, *Opuntia ficus-indica*. In our collections, some samples of necrotic cladodes or fruits contained pupae of both species; while others had only one of the species. We estimated the average distance to nearest neighbor pupa of the same species in single and mixed pupa

Table 1 Pupa aggregation in five species of *Drosophila* in the wild

Species of *Drosophila*	Type and no. of breeding sites	Mean number of pupae per breeding site		Mean aggregation index per breeding site	
		Mean	Variance	Mean	Variance
D. simulans	Apricot ($N = 44$)	19.70 ($N = 868$)	30.80	0.13*	0.15
	Prickly pear				
	Fruits ($N = 33$)	23.00 ($N = 759$)	30.60	0.17*	0.30
	Tissue ($N = 18$)	4.82 ($N = 87$)	23.14	0.11*	0.19
	Red grape ($N = 123$)	6.79 ($N = 835$)	12.78	0.06*	0.21
D. buzzatii	Prickly pear				
	Fruits ($N = 33$)	31.40 ($N = 1036$)	46.30	0.09*	0.08
	Tissue ($N = 18$)	12.00 ($N = 216$)	11.30	0.24*	0.29
D. melanogaster	Pear ($N = 28$)	19.78 ($N = 560$)	17.42	0.16*	0.04
D. immigrans	Strawberry ($N = 28$)	3.96 ($N = 111$)	1.05	0.12*	0.02
D. hydei	Prickly pear fruits ($N = 12$)	0.42 ($N = 5$)	0.78	0.01*	0.00
	Apple ($N = 25$)	3.40 ($N = 85$)	1.23	0.14*	0.08

Aggregation was estimated by using Clark and Evans (1954) aggregation index. The pupae were detected on the indicated breeding sites

* $P < 0.05$

aggregations of equal density (N fluctuated between 50 and 100 pupae), and *R*-values were compared using ANOVA (Clark and Evans 1954).

We also calculated Ripley '*K* function for mixed pupa aggregations of *D. simulans* and *D. buzzatii*. The *K* function is defined as the expected number of points of pattern 2 (for instance, number of *D. simulans* pupae) within a given distance *r* of an arbitrary point of pattern 1 (in this case pupa aggregations of *D. buzzatii*), divided by intensity λ_2 of points of pattern 2 (Diggle 1983). Square-root transformation of *K*, called L-function, is used in the calculations. $L_{12}(r)$ values > 0 indicate that there are on average more points of pattern 2 within distance *r* of points of pattern 1 than would be expected under independence. $L_{12}(r)$ values < 0 indicate that the two patterns of pupae are separated at distance *r*. We calculated $L_{12}(r)$ for distances between pupae of $r = 1.0$cm, 1.5 cm and 2.0 cm. We selected these distances based upon the relative sizes of *D. simulans* and *D. buzzatii* pupae.

Additional analysis

Subjects and larval recognition

We used wild type *D. buzzatii* (subgenus *Drosophila*) and *D. simulans* (subgenus *Sophophora*) strains to investigate whether larval response to conspecific and heterospecific larvae influences pupation site selection. The stocks were established with flies from a mixture of 10 males and 10 females that emerged from five pieces of rotten prickly pear cladodes and from five decaying fruits (i.e., a total of 200 individuals) collected in Til–Til (see above). The flies were kept in half pint bottles containing 50 cc of Burdick's medium (1954) at 24°C.

Conspecific and heterospecific larval recognition

We used two experiments to test the response of *D. buzzatii* and *D. simulans* larvae to odors of conspecifics and heterospecific larvae. In the first experiment, one 2.0 × 2.0 cm piece of paper was moistened for 1 h in Burdick's (1954) culture medium occupied by *D. buzzatii* (or *D. simulans*) larvae, while another paper of the same size was moistened for a similar time in "virgin" Burdick's medium. In the second experiment, one of the papers was moistened in Burdick's medium occupied by *D. buzzatii*, and the other in medium occupied by *D. simulans*. The papers were placed on opposite sides of Petri dishes filled with 3% agar gel to a depth of 2 cm. Fifty-third instar larvae of *D. buzzatii* (or *D. simulans*) were placed on the center of each of the Petri dishes at a distance of 3.0 cm from each of the papers; and the number of larvae on each one was recorded every 5 min up to 30 min. Each experiment was replicated eight times.

Larval response to acidity/alkalinity

Using the same fly stocks, we also examined the reaction of *D. buzzatii* and *D. simulans* larvae to changes in acidity/alkalinity within fruits of *O. ficus-indica*. For each of the species, a set of eight Petri dishes was filled with 3% agar gel to a depth of 2 cm. The agar surface was divided into three zones of equal size leaving in the centre a circle of 2 cm of diameter. We covered one zone with a film of decaying fruit of prickly pear at pH 4.5; we spread a film of fruit at pH 7.0 over the second zone, and the third was covered with fruit at pH 8.5. We used the same fruit in all sixteen trials. Fifty-third instar *D. buzzatii* (or

D. simulans) larvae were deposited onto the center circle, and the number of larvae on each zone was recorded every 5 min up for 30 min.

Statistical analysis

We used a Mann–Whitney U test (Sokal and Rohlf 1995) to compare the mean distance between nearest neighbor *D. simulans–D. simulans,* and *D. buzzatii–D. buzzatii* pupae versus mean distance between *D. simulans–D. buzzatii* and *D. buzzatii–D. simulans* pupae. We also examined homogeneity for replicates within larval responses to odors of other larvae, and larval reactions to acidity/alkalinity using a variance analysis between replicates within a recording time. We did not find a significant heterogeneity among replicates, and thus we pooled data over replicates. We used G-test of independence to estimate significance of the larval reactions to odor of other *Drosophila* larvae and pH test at 30 min of observation period.

Results

Breeding site collections and nearest neighbor analysis in the wild

Pupae of all the species were aggregated in the seven different breeding sites (aggregation indexes (R) < 1.0; $Z \gg 1.96$, $P < 0.05$ (Table 1). Parameter dw (Diggle's G function) values were all positive confirming that pupae of the indicated species form aggregations on the substrates (*D. melanogaster*, $dw = 0.25$, $P < 0.05$; *D. simulans*, $dw = 1.01$, $P < 0.05$; *D. buzzatii*, $dw = 0.86$, $P < 0.05$; *D. hydei*, $dw = 0.93$, $P < 0.05$; and *D. immigrans*, $dw = 0.74$, $P < 0.05$).

Aggregation of *D. simulans* and *D. buzzatii* pupae was independent of the number of pupae in the sample (aggregation indexes (R) < 1.0; $Z \gg 1.96$, $P < 0.05$; Fig. 1).

In fruits containing pupae of both *D. buzzatii* and *D. simulans* each species aggregated preferentially with conspecifics. Indeed, mean distance between nearest neighbor pupae.

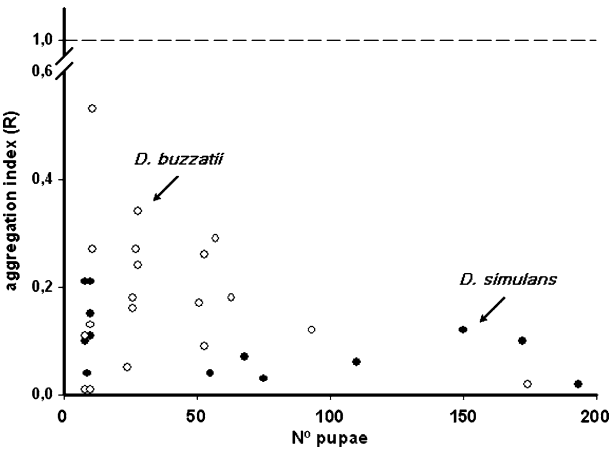

Fig. 1 Aggregation index (R) and number of pupae of *D. buzzatii* and *D. simulans* on necrotic prickly pear fruits (*O. ficus-indicus*). *Dotted line* ($R = 1.0$) indicates that individuals are randomly distributed over substrates, $R = 0.0$ indicates maximum aggregation. *D. buzzatii*, *white circles*; *D. simulans*, *black circles*

D. buzzatii–D. simulans is 4.42 times greater than between nearest neighbor pupae *D. buzzatii–D. buzzatii* (Mann–Whitney *U*-test, $P < 0.05$; Fig. 2), and the mean distance between nearest neighbor pupae *D. simulans–D. buzzatii* is 2.60 times greater than between nearest neighbor pupae *D. simulans–D. simulans* (Mann–Whitney *U*-test, $P < 0.05$; Fig. 2). Moreover, our analysis using Ripley's K function indicated that fewer heterospecific pupae were present in the neighborhood of a focal pupa than would be expected under assumption of independence ($L_{12(r\,=\,1.0)} = -0.23 \pm 0.07$; $L_{12(r\,=\,1.5)} = -0.03 \pm 0.01$, and $L_{12(r\,=\,2.0)} = -0.006 \pm 0.002$. Collectively, the results of these analyses suggest that *D. buzzatii* and *D. simulans* tend to group more closely with conspecific in mixed species aggregations.

Recognition of conspecific and heterospecific larvae

In both odor experiments, *D. buzzatii* larvae preferently moved toward papers that had been exposed to food occupied by *D. buzzatii* larvae. In the first experiment, at 30 min of observation, 65.00% of *D. buzzatii* larvae were on the paper from the *D. buzzatii* medium, while 1.00% were on the paper from virgin medium (G-test of independence, $\chi^2 = 38.67$, df = 1, $P < 0.05$ (Fig. 3a). About 34% of the larvae were on agar going in and going out of the *D. buzzatii* paper. In the second experiment, at 30 min of observation, 50.00% of *D. buzzatii* larvae were on *D. buzzatii* paper, while 5.00% were found on *D. simulans* paper (G-test of independence, $\chi^2 = 11.39$, df = 1, $P < 0.05$ (Fig. 3c).

In the first experiment, *D. simulans* also preferred the paper exposed to food in which conspecifics had been reared. Thus, at 30 min 20.00% of the larvae were on filter paper

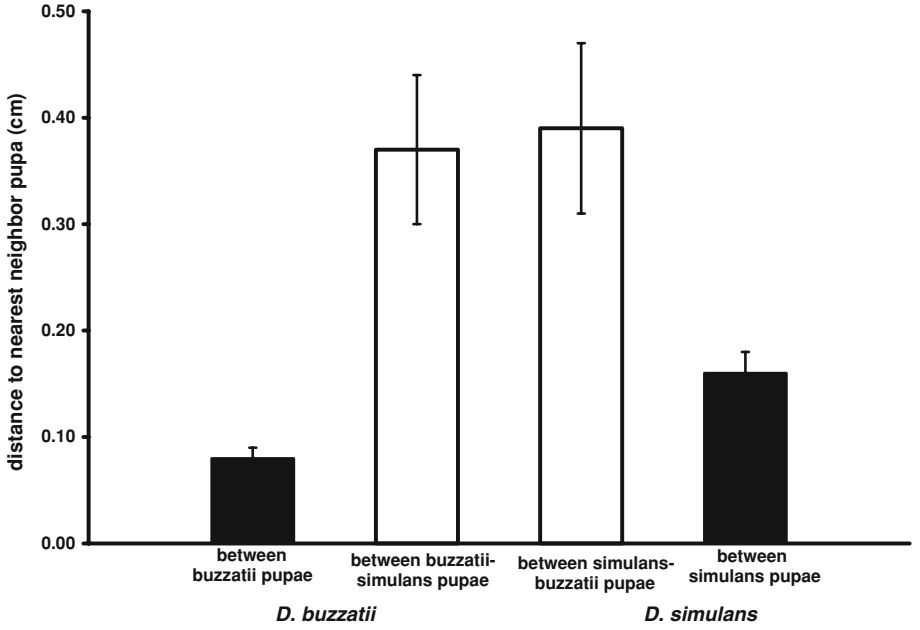

Fig. 2 Mean distance (cm) between nearest neighbor pupae *D. buzzatii* and *D. simulans*. *White column*, distance between conspecific pupae. *Black column*, distance between heterospecific pupae. *N* of pupa pairs = 160 (*buzzatii–buzzatii*); 160 (*buzzatii–simulans*); 114 (*simulans–simulans*); and 114 (*simulans–buzzatii*)). The pupae were found in the wild on decaying prickly pear fruits

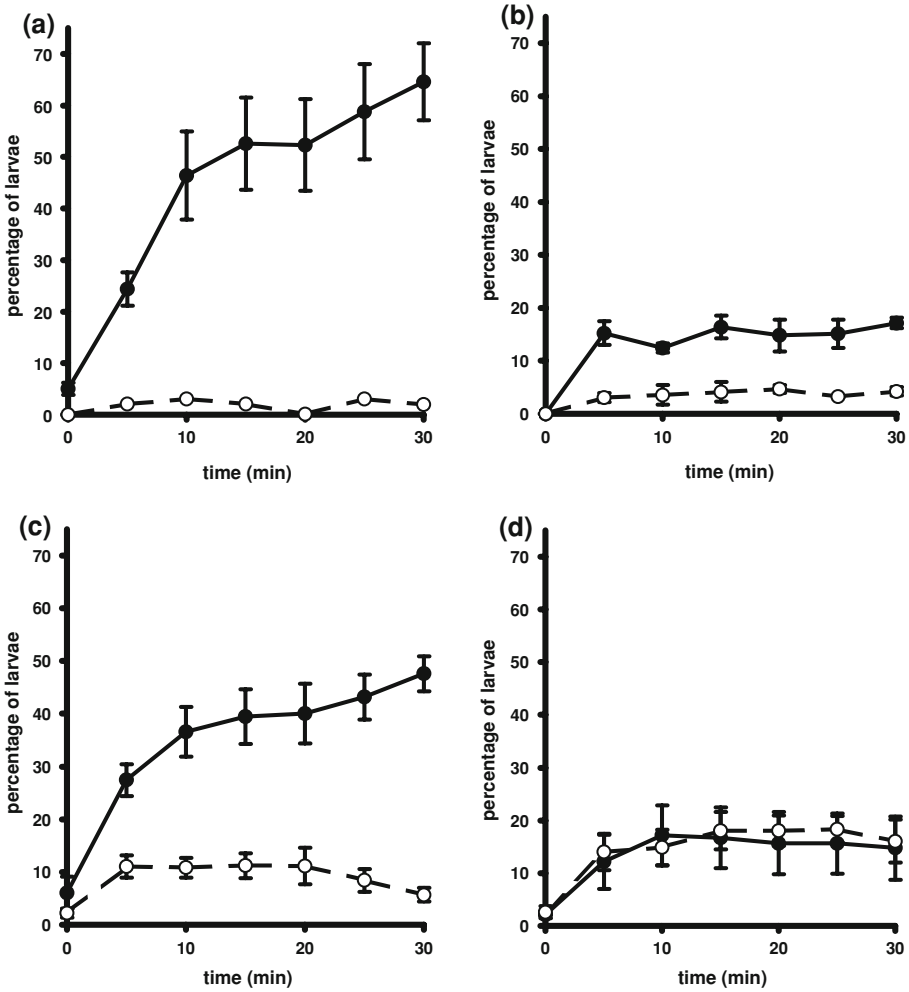

Fig. 3 **a–d** Reactions of larvae of *D. buzzatii* and *D. simulans* to larval odors. The larvae were given a choice between a paper soaked in culture medium that was either occupied by conspecifics or was "virgin" medium (**a**, **b**). In a second experiment the larvae were given a choice between paper soaked in culture medium occupied by either conspecifics or heterospecifics (**c**, **d**). Larvae on the paper of their own species, *black circles*. Larvae on the other type of paper, *white circles*. Each experiment was replicated eight times. $N = 50$ for replicate

from *D. simulans* media, whereas 1.00% were on the paper from "virgin" medium (G-test of independence, $\chi^2 = 12.53$, df $= 1$, $P < 0.05$ (Fig. 3b). About 79% of the larvae remained near the *simulans* paper with a few larvae going in and going out of the paper.

However, in the second experiment *D. simulans* larvae showed no clear preference. Thus, at 30 min 17.00% of *D. simulans* larvae were found on each of the papers (G-test of independence, $\chi^2 = 0.98$, df $= 1$, NS, (Fig. 3d). Together, results from these experiments suggest that *D. buzzatii* larvae are clearly attracted to conspecifics. *D. simulans* larvae prefer areas occupied by other larvae but do not differentiate between conspecifics and heterospecifics.

Larval response to acidity/alkalinity

Results of *D. buzzatii* larval response to variation in pH of fermenting tissues revealed that at 30 min there were more larvae on food at pH of 8.5 than on food at either pH = 7.0 or pH = 4.5 (G-test of Independence was $\chi^2 = 15.87$, df = 2, $P < 0.05$). In the case of *D. simulans* larvae, at 30 min about 38.00% were found on tissue at pH = 8.5, while 24% of larvae were on the food either at pH = 7.0 or pH = 4.5 (Fig. 4b; G-test of Independence value was $\chi^2 = 9.02$, df = 2, $P < 0.05$). Thus, *D. buzzatii* and *D. simulans* larvae of third instar show preferences for alkaline substrates to pupate.

Discussion

Environmental patchiness has importance for the persistence of communities of interacting species (Atkinson and Shorrocks 1984). One important feature is the extent to which individuals of different species are aggregated over patches. Our results obtained in the field show that pupae of five *Drosophila* species are aggregated in naturally occurring

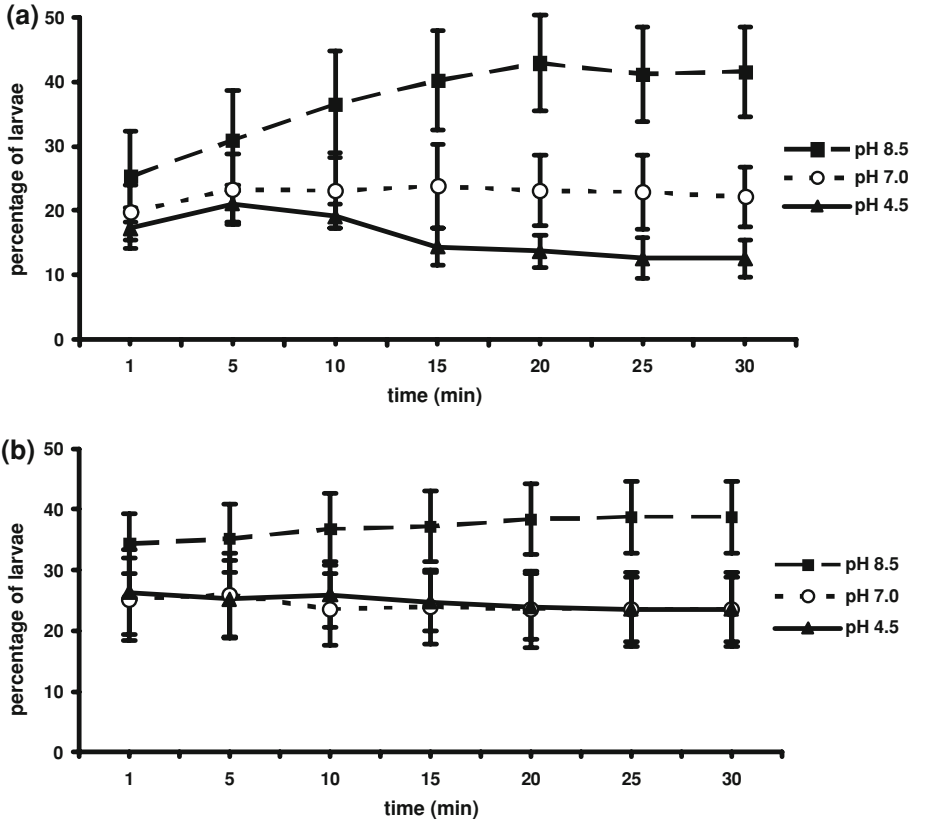

Fig. 4 Reaction of *D. buzzatii* (**a**) and *D. simulans* (**b**) larvae to acidity/alkalinity. Larvae chose between decaying prickly pear fruits at pH 8.5 (*black squares*), 7.0 (*white circles*) and 4.5 (*black triangles*). The experiment was replicated eight times for each species. $N = 50$ for replicate

breeding sites, and that aggregations occur irrespective of density of pupae. In the Central Valley of Chile a variety of decaying fruits are available to species of *Drosophila* (Brncic 1987). Although they are abundant, the discrete nature and small size of these rearing environments inevitably result in interactions among conspecific and heterospecific larvae. In such cases, *D. buzzatii* and *D. simulans* form distinct pupal aggregations when both species are present in a single breeding patch (Fig. 2). We have also observed pupa aggregations of the same species in the case of *D. melanogaster* and *D. simulans* that coexist in the wild in the same decaying grape unit (unpublished observations). Similar results have been reported for *D. melanogaster*, *D. hydei* and *D. pavani* under laboratory conditions (Medina-Muñoz and Godoy-Herrera 2004), but this study is the first to show such aggregation behavior in the wild.

Our experiments based in larval recognition of conspecifics and heterospecifcs suggest that pupa aggregations observed in the wild are based in part on response to odors emanated from other larvae. Indeed, third instar *D. buzzatii* larvae move toward conspecific odors which could lead to pupa aggregations (Fig. 3a, b). In contrast, while *D. simulans* larvae are attracted to the odors of other larvae, they do not appear to distinguish conspecifics and heterospecifics. However, it is interesting to remind that about 79% of the larvae were near by the *simulans* paper, suggesting that they could recognize odors of their species only that they continuously are go in and go out the paper. In fact, in nature we found that *D. simulans* tends to form conspecific pupal aggregations. One explanation for this might be that *D. simulans* larvae have a shorter larval period (4 days) than those of *D. buzzatii* (8 days) at 24°C, meaning that *D. simulans* larvae that leave the breeding site encounter only conspecific larvae and pupae outside the breeding site. Recognition may have occurred when first and second instar larvae of the two species were feeding in the same tissue. We have individually reared larvae of *D. simulans* and *D.buzzatii* in isolation from other conspecifics. These larvae were tested for recognition of conspecific and heterospecific larvae in odor experiments described in this work. We found that these larvae form puparia randomly with respect to conspecific and heterospecific (unpublished data). Thus, some type of chemosensory conditioning might perhaps to be involved in the formation of *Drosophila* pupa aggregations.

Third instar larvae of *D. buzzatii* and *D. simulans* tend to also prefer alkaline substrates to pupate (Fig. 4a, b). This preference is remarkable in *D. buzzatii*, and it is consistent with the finding that in the wild the decay process within prickly pear fruits leads to alkalinity. We have found decay prickly pear tissues at pH = 12.8 and pH = 13.2 (unpublished data), and thus the observed preferences are not entirely unexpected. Furthermore, at least in *D. melanogaster*, fitness components such as development time and viability are negatively affected by acidity (Hodge and Caslaw 1998). Our data also indicate that there are quantitative differences in larval distribution of *D. simulans* and *D. buzzatii* at the pH levels (Fig. 4a, b). Both species prefer alkaline substrates to pupate, but they differ in percentage of larvae at pH 7.0 and 4.5. In *D. simulans*, at 30 min, percentage of larvae on food at pH 7.0 and 4.5 was similar, but in *D. buzzatii* larvae very different percentages of larvae were recorded at these pH (Fig. 4a, b). These results suggest that the two species utilize different strategies when searching for pupation sites. *D. buzzatii* has been collected in four out of six biogeographic regions, and it breeds principally on decaying tissue and fruits of *Opuntia* (Powell 1997). *D. simulans* has been collected in the six biogeographic regions, and its larvae exploit a wider variety of fermented resources (Shorrocks 1982). These ecological differences among the species no doubt in part underlie larval preferences for pupation on substrates with different degree of acidity/alkalinity.

In summary, the data suggest that responses to conspecific and heterospecific larvae and to differences in the chemical characteristics of breeding substrates are important factors in selection of pupation sites in *Drosophila*. The genetics bases of these behaviors are unclear, however, and their neurobiology is almost unknown (de Bono and Sokolowski 2007). Clearly, neurogenetic studies using neurological mutants on these and other larval behaviors are necessary to understand properly how *Drosophila* larvae select pupation sites in the wild.

Selection of the appropriate pupation sites is a critical step in the life cycle of *Drosophila* and other holometabolous insects. In addition to factors such as predation and desiccation avoidance, our data suggest that pupation site selection in *Drosophila* contributes to niche separation between species that breed in the same site. Studies of natural populations of *D. melanogaster*, *D. hydei* and *D. pavani* have also shown that aggregation of pupae increases in the presence of another *Drosophila* species (Medina-Muñoz and Godoy-Herrera 2004). We suggest that larval behaviors reported here for *D. buzzatii* and *D. simulans* may serve as a general model system to understand the evolution and ecology of *Drosophila* larvae in their natural breeding sites.

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