The Neuro-Ecology of *Drosophila* Pupation Behavior

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Abstract

Many species of *Drosophila* form conspecific pupa aggregations across the breeding sites. These aggregations could result from species-specific larval odor recognition. To test this hypothesis we used larval odors of *D. melanogaster* and *D. pavani*, two species that coexist in the nature. When stimulated by those odors, wild type and vestigial (vg) third-instar larvae of *D. melanogaster* pupated on conspecific larval odors, but individuals deficient in the expression of the odor co-receptor Orco randomly pupated across the substrate, indicating that in this species, olfaction plays a role in pupation site selection. Larvae are unable to learn but can smell; the syn97cs and rut strains of *D. melanogaster*, did not respond to conspecific odors or *D. pavani* larval cues, and they randomly pupated across the substrate, suggesting that larval odor-based learning could influence the pupation site selection. Thus, Orco, Syn97cs and rut loci participated in the pupation site selection. When stimulated by conspecific and *D. melanogaster* larval cues, *D. pavani* larvae also pupated on conspecific odors. The larvae of *D. gaucha*, a sibling species of *D. pavani*, did not respond to *D. melanogaster* larval cues, pupating randomly across the substrate. In nature, *D. gaucha* is isolated from *D. melanogaster*. Interspecific hybrids, which result from crossing *pavani* female with *gaucha* males clumped their pupae similarly to *D. pavani*, but the behavior of *gaucha* female x *pavani* male hybrids was similar to *D. gaucha* parent. The two sibling species show substantial evolutionary divergence in organization and functioning of larval nervous system. *D. melanogaster* and *D. pavani* larvae extracted information about odor identities and the spatial location of congeners and alien larvae to select pupation sites. We hypothesize that larval recognition contributes to the cohabitation of species with similar ecologies, thus aiding the organization and persistence of *Drosophila* species guilds in the wild.

Introduction

No animal lives in isolation. Animals interact with one another in many different ways using their sensory systems. Olfaction and gustation help to detect, localize and recognize congeners and heterospecifics, and provide information regarding food availability and the chemical features of environments where animals live [1–11]. Holometabolous insect larvae possess sophisticated olfactory and gustatory receptors and brain structures that process a variety of olfactory and gustatory inputs [1–9,12,13], suggesting that the importance of these sensory systems for the ecology and evolution of these individuals. Larva behavior is relevant to understand certain aspects of holometabolous insect population biology, including spatial distributional patterns, interactions between congeners and alien individuals, and feeding preferences. However, in *Drosophila* there are considerable gaps in our understanding of larva behavior because the ecology of natural breeding sites in which neurological organization functions is poorly known. Additional studies are needed because several investigations have suggested that pupation site selection depends on discrimination of chemicals emitted by congeners and alien larvae [8,14,15]. Pupation behavior is important for fitness because it implies habitat use and plays a role in the coexistence of species with similar ecologies. Experiments addressing these problems provide insight into how the nervous system generates behaviors related to recognition between conspecific and alien larvae, and how the behaviors are genetically specified and linked to neural circuits. These two aspects are key to understand the evolution of behavior in the genus *Drosophila*.

To address the above problems we observed the behavior of *Drosophila* larvae in nature. We also identified species cohabiting within decaying fruits (grape, apple, peach and prickly pear) and cactus cladodes (*Opuntia ficus-indica*). We noted that many *Drosophila* species formed conspecific pupa aggregations across the breeding sites away from the pupae of other species (see Figures S1 and S2). These observations suggest that larvae can recognize congeners and alien larvae. Namely, larvae appear to associate spatial position of congeners with favorable locations to pupate while eluding pupae and/or larvae of other species.

*Drosophila* breeding sites are ephemeral and variable and their ecological conditions can change quickly [16]. Microbial action on sugars in commercial fruits as apple, peach, and prickly pear, and on plant tissues produces variable concentrations of odors composed of alcohols, esters and some fatty acids [10,17,18]. In these circumstances, species-specific larval odors could act as indicators of spatial position of conspecific and alien larvae. Namely, these chemical cues would provide information on identity and emission source, thus orienting larval movements within the breeding sites. Identifying congeners would be more efficient if the process was coupled with social odor learning. For individuals who reside in changing environments, learning has a profound effect on their fitness [9]. Reviews of the factors that
contribute to the emergency of learning emphasize the role played by changing habitats [19,20]. Namely, learning is favored by natural selection when environmental changes occur in a short lapse of time, and those changes are recurring [21]. Learners may increase their fitness acquiring behaviors similarly to those expressed by other members of the species. These behaviors will be adaptive if they are appropriate for the current environmental state [22]. The relationship between pupation behavior in ephemeral breeding resources and social odor learning in Drosophila has received little attention. Previously, we observed that Drosophila simulans and Drosophila buzzatii larvae that bred with congeners formed pupa aggregations, but larvae that were individually reared in isolation from the conspecifics pupated randomly across the substrata [8,14]. These results suggested larval social odor learning. Here we describe pupation behavior of D. melanogaster mutant larvae that cannot smell and cannot learn via conspecific and D. pavani larval odors. In Chile, these two species use decaying fruits of Opuntia ficus-indica as breeding sites. However, there are also closely related Drosophila species that differ in their physiological ability to use different foods, and this may have consequences for the larva behavior [23]. Larvae of those species that exploit a range of breeding sites have more opportunities to be reared with other Drosophila species than larvae of ecologically restricted species [16]. The presence/absence of other species might be an evolutionary pressure to Drosophila larval nervous system. D. pavani and Drosophila gaucha are allopatric sibling species in the mesophragmatica group that reside in Chile and Argentina, respectively [24–26]. In Chile, D. pavani and D. melanogaster emerge from decaying prickly pear fruits. In Argentina, D. gaucha breeds on decaying prickly pear cladodes (tissue). D. melanogaster larvae die when they are transferred to decaying cladodes (unpublished data). D. gaucha dies when it is bred on prickly pear fruit. Thus, D. gaucha and D. melanogaster larvae are ecologically isolated. We investigated pupation behavior of D. pavani and D. gaucha stimulated by conspecific and D. melanogaster larval cues.

Materials and Methods

Subjects

We tested wild type larvae of natural Chilean populations (Til-Til and Trana strains) and laboratory stocks (Oregon R-c and Canton – Special strains) of D. melanogaster. We also examined larvae of the vestigial (vg) strain. The Oregon R-c and vg strains differ in some larval behaviors. For example, Oregon R-c larvae dig deeper into the substratum than vg larvae [27]. We also tested three neurological mutants derived from the Canton-Special (CS) strain of D. melanogaster. Larval perception of odorants in the Orco mutant strain has been blocked because the dendritic localization of the receptors has been eliminated. Thus, the Orco mutation disrupts behavioral and electrophysiological responses to many odorants [29]. The Syn97CS mutation affects presynaptic vesicle release in the entire larval brain, and olfactory associative learning is reduced in approximately 50% with respect to CS larvae; however, responsiveness to stimuli and motor performance in untrained animals are normal [28]. The rut locus participates in olfactory conditioning learning in D. melanogaster and it is expressed in neurons located in larval and adult mushroom bodies; rut does not affect larval locomotion and responsiveness to stimuli [30].

In 1952, Professor Danko Brncic brought the Oregon R-c and vg strains from Columbia University (USA) to the University of Chile. Dr Bertram Gerber, University of Würzburg in Germany sent the Canton-Special (CS), Orco, Syn97CS and rut strains to our laboratory. The wild type Trana strain of D. melanogaster was
established with 22 adults that emerged from 20 decaying grapes (Vitis vinifera, Pais variety); Trana is located 380 km southwest of Santiago. The wild type Til-Til strain of D. melanogaster was formed with 18 adults that emerged from three decaying prickly pears (Cactus sp.) collected in Til-Til, which is 50 km northwest of Santiago. The Til-Til and Trana larvae in the laboratory experiments were the fourth generation.

To investigate larval olfactory recognition in other Drosophila species, we examined the endemic South American sibling species D. pavani and D. gaucha (Subgenus Drosophila, mesophragmatica group) and the F1 reciprocal hybrids [24–26]. The mesophragmatica group comprises many South American species and forms a phyletic unit [24]. D. pavani is predominantly Andean in distribution, whereas D. gaucha is distributed in Argentina, Uruguay and Southern Brazil. Both species share characteristics with domesticated species in that the adults can be collected from orchards, gardens, and other plants associated with human activities [26]. Under laboratory conditions, the two species can produce viable but sterile hybrids [31]. D. pavani and D. gaucha have similar development durations for molting, wandering and pupating [24–26]. D. pavani and D. gaucha require approximately one and half year to assimilate to laboratory conditions. For this reason we tested the La Florida and Buenos Aires strains (see below).

In 1978, Professor Danko Brncic founded the La Florida strain of D. pavani with approximately 18 individuals collected from banana traps in La Florida, Santiago (Chile). Same prickly pear plants remain in this neighborhood today. In 1989, Dr Esteban Hasson sent the Buenos Aires strain of D. gaucha to us. This strain was created with 10 adults collected from banana traps in Buenos Aires (Argentina). The traps were established near prickly pear plants. The sex ratio was maintained for the all strains.

The strains were all maintained by mass culture at 24 ±1°C, 70% humidity (D. melanogaster) and 18 ±1°C, 80% humidity (D. pavani and D. gaucha). D. pavani and D. gaucha grow better at this temperature and humidity than at 24°C. All stocks were maintained under constant light, because facilities to change the light/dark period were not available in the laboratory.

Croses

Fifteen-day-old D. pavani (La Florida strain) and D. gaucha (Buenos Aires strain) males and females were reciprocally crossed. At this age, individuals are sexually competent [24]. Homogametic mating within strains also served as controls for the interspecific crosses. Crosses between the La Florida (D. pavani) and Buenos Aires (D. gaucha) strains provided abundant hybrid larvae of the two sexes [31].

Larva collection

Groups of 40–50 inseminated females of D. melanogaster, D. pavani, D. gaucha, and D. pavani females and D. gaucha females crossed with males of the other species were allowed to oviposit for 2–3 h on plastic spoons containing the culture medium. Thirty eggs of the species, strains and hybrids were randomly collected with a dissecting needle. Each batch of eggs was incubated on fresh spoons for 96–100 h at 24°C (D. melanogaster strains) and 168–172 h at 18°C (D. pavani, D. gaucha and the hybrids). D. melanogaster larvae emerged after 2–4 h. D. pavani, D. gaucha and hybrid larvae emerged 48 h after the eggs were laid. The culture medium was supplemented daily with 40% fresh baker’s yeast paste. One hour before an experiment, third-instar larvae were collected from the glass wall of rearing vials, washed twice with distilled water, and identified by the presence of protruded anterior spiracles [32]. All larvae were raised in half-pint bottles at 24°C (D. melanogaster) and 18°C (D. pavani and D. gaucha) on Burdick’s medium [33].

Larval odor recognition and pupation site selection

We employed three treatments to test the response of D. melanogaster larvae to conspecific and D. pavani larval odors. In the first treatment, one 2.0 x 2.0 cm piece of Whatman cellulose filter paper was moistened for 1 h in Burdick’s medium used for 4–5 days by D. melanogaster (or D. pavani) larvae, whereas another identically sized filter paper was moistened in sterile Burdick’s medium for a similar period of time. Before transferring the two filter paper types to Petri dishes, each piece of paper was carefully examined under stereomicroscope to verify that no food was adhered to the surface. In the second treatment, one piece of paper was impregnated for 1 h with food worked by larvae of the strain (Canton-Special, Til-Til, Trana, vestigial, Orco, Syn97CS and rut strains of D. melanogaster). The other filter paper was moistened for a similar time with food processed by the Oregon R-c larvae. The test for larvae of this strain was an Oregon R-c filter paper, and a Canton – Special filter paper. In the third treatment, the larvae were placed together with a filter paper that had been moistened for 1 h in food occupied by larvae of D. melanogaster strains, and another moistened for a similar time with the food used by D. pavani larvae. These treatments were also applied to D. pavani, D. gaucha and the interspecific reciprocal hybrids.

For each treatment, 10-cm Petri dishes were filled with 10 ml of 3% agar gel. Each filter paper type was deposited onto agar in Petri dishes on opposite sites at 6 cm one from the other. Batches of 20 third-instar larvae of each species and strain were introduced into corresponding Petri dish and gently deposited onto the middle of the agar 3 cm from each piece of paper. Once the larvae actively moved, we transferred the Petri dishes to the culture room maintained at 24 ±1°C (D. melanogaster), and 18 ±1°C (D. pavani, D. gaucha and the hybrids). To decrease possibilities that temperature differences and illumination conditions would interfere with pupation site preferences, we always deposited the Petri dishes in the identical location in the culture room. The number of pupae on/under each paper was recorded two (D. melanogaster), four–five days later (D. pavani, D. gaucha and the hybrids). Some pupae were observed on the agar surface near the paper. Those individuals detected within 10 mm of the border were counted as belonging to that paper. Replicate measurements (10 measurements, 200 larvae) were performed for each strain by dividing the larvae into groups of 5 replicates and conducting the measurements in parallel. Thus, we were able to estimate magnitude of environmental odor variation on larval response to chemical cues. We did not know whether the substances present in the paper could diffuse through the agar, which might have introduced an additional source of experimental error to our measurements because of...
possible larval gustatory responses. To address this variable, we tested Orco mutant larvae. There were no odor-evoked responses for the specialized olfactory sensory neurons of Orco larvae. The Orco mutation does not affect gustatory neurons [29].

We also monitored the pupa distributions on the agar in the virgin food/D. pavani food and virgin food/Oregon R-c food treatments. In the first treatment, wild type and vg pupae of D. melanogaster were clumped across the agar, whereas Orco, Syn97CS and rut pupae were randomly distributed. In the virgin food/Oregon R-c food treatment, wild type, vg, Orco, Syn97CS and rut pupae were scattered across the agar. We calculated the Clark and Evans aggregation index [34] to compare the pupa distributions in the two treatments. We estimated the average distance to the nearest neighboring (rA) pupa using a 0.5-cm Cartesian grid and then compared this average value with the expected value (rE) for the identical number of individuals randomly distributed on an area of equal size (rE = ½πρ, where ρ is the pupa density. The ratio R = rA/rE, reflects the spatial distribution of the individuals (aggregated, random, over-dispersed) with values ranging between R = 0.0 (maximum aggregation) and R = 2.15 (uniform). When individuals are randomly distributed, R = 1.0 [34]. We followed a similar procedure for the pupa distributions of D. pavani (La Florida strain), D. gaucha (Buenos Aires strain) and the reciprocal interspecific hybrids in the virgin food/D. pavani (D. gaucha, hybrid) food, and virgin food/Oregon R-c food treatments.

All behavioral tests were performed between 10:00 and 14:00 hours under controlled temperature, 22.00±1.0°C, light, 6.2±0.2×10⁻³ lx, and humidity, 70±3%.

Statistical analysis

We tested for homogeneity between replicates within a treatment and strain. We applied a G-test of Independence to compare the pupa percentages: N = 10 replicates per strain, N = 8 strains of D. melanogaster, N = 3 treatments. The test was also applied to the pupa percentages obtained for replicated experiments: N = 10 replicates within a treatment, N = 2 treatments, with D. pavani, D. gaucha, and hybrid larvae, N = 4 genotypic groups. None of the individual chi-square tests detected a significant deviation from the hypothesis of homogeneity between the replicates within a strain or treatment (the Chi-square values were all lower than the critical value χ² = 23.59, df = 9, P = 0.05; Table S1). Thus, we pooled the replicate data within treatments, strain, and species.

We also applied a binomial test to the data. The null hypothesis stated that there was no difference between the probability of selecting one filter paper type and the probability of selecting another paper within a treatment or strain (p = q = ½). The binomial test was selected because the data were in two discrete categories and the experimental design utilized one sample (see above homogeneity between replicates within a treatment and strain and Table S1). The rejection region of the null hypothesis was equal or less than α = 0.01.

An analysis of variance was applied to compare pupa aggregation indexes (R) of D. melanogaster in the presence and absence of D. pavani larval volatiles. The analysis was also applied to pupa aggregations of D. pavani, D. gaucha and the hybrids stimulated by D. melanogaster larval volatiles.

Results

Pupation site selection: D. melanogaster

More than 60% of wild type and vg pupae of D. melanogaster were on the conspecific paper, thus indicating that they were stimulated by odors emanating from the two types of paper. Pupation of sterile food and the other with food processed by larvae of the own strain. (Fig. 1 A-E). Differences in pupa percentages on the two paper types are statistically important (Table S2). By contrast, with paper moistened with virgin food and food worked by D. pavani larvae more than 60% of the wild type and vg D. melanogaster pupae were located on the agar, and approximately 17% were located on the two paper types (Fig. 1 I-M, and Table S2).

In the two treatments, more than 60% of the Orco pupae were scattered across agar (Fig. 1 F-N, and Table S2), thus suggesting that the Orco larvae did not detect the odors emitted from the two paper types. In a similar way, stimulated by conspecific and D. pavani larval odors, Syn97CS and rut larvae also were randomly distributed across agar (Fig. 1 N-P, and Table S2). Thus, the Orco, Syn97CS and rut loci are necessary for pupation site selection in D. melanogaster.

The pupa aggregation (R) indexes were calculated for the wild type and mutant strains of D. melanogaster. In the virgin food/D. pavani food treatment (Fig. 2 A-H), the aggregation R-values for the wild type and vg strains of D. melanogaster were approximately R = 0.0, which indicated grouped pupae (Fig. 2 A-E, black circles). In the presence of chemicals emanating from the two types of papers moistened with virgin food and Oregon R-c food, the calculated R-values were approximately R = 1.0, which indicated that the pupae were randomly distributed across the substrate (Fig. 2 A-E, white triangles). These differences in the pupa aggregation indexes were statistically important (ANOVA, Table S3). In the presence of conspecific and alien larval odors, the pupa aggregation (R) indexes of the Orco, Syn97CS and rut strains were approximately R = 1.0, which confirmed that the larvae pupated randomly across the agar (Fig. 2 F-H, and Table S3). The results again indicated that these three loci are necessary for D. melanogaster pupation behavior. For all strains tested, the aggregation indexes were independent of the number of pupae on the agar (Fig. 2 A-H).

Fig. 3. A-E shows that D. melanogaster larvae that were stimulated by conspecific odors used the two types of paper to pupate. However, in the presence of conspecific and D. pavani larval cues, the wild type and vg D. melanogaster pupae were preferentially observed on the conspecific paper (Fig. 3 I-M; Table S2). In the two treatments, similar percentages of Orco, Syn97CS and rut pupae were found on the two paper types (Fig. 3 F-H and N-P; Table S2). These results and those in Figs. 1 and 2 again indicate that in D. melanogaster olfaction is required for pupation site selection and that genes expressed in the neurons of the peripheral olfactory circuits [29], synapses [11], and mushroom bodies [2] participate in D. melanogaster pupation behavior.

Pupae were also detected outside the two paper types on the Petri dish agar. In the treatment with two types of conspecific food (Fig. 3 A-H), a few pupae were present on agar. The calculated (R) aggregation indexes (N = 10 replicates) were (i) Oregon R-c, 0.88 ± 0.02; (ii) Canton-Special, 0.83 ± 0.09; (iii) Til-Til,
0.70 ± 0.04; (iv) Trana, 0.91 ± 0.05; (v) vg, 0.79 ± 0.06; (vi) Orco, 0.81 ± 0.08; (vii) Syn97CS, 0.95 ± 0.10; and (viii) rut, 0.97 ± 0.05. In the conspecific food/D. pavani food treatment (Fig. 3 I–P), 79 ± 2.31% of the wild type and vg D. melanogaster pupae were adhered one another, as observed in nature (Figs. S1 and S2). In these two identical treatments, the Orco, Syn97CS and rut pupae were scattered across the substrate. These results are consistent with those in Figs. 1–3.

Pupation site selection: the sibling species D. pavani and D. gaucha

In the virgin food/conspecific food treatment, approximately 68% of D. pavani and the pavani female x gaucha male hybrid larvae pupated on the conspecific paper (Fig. 4 A, C, Table S2). In this identical situation, D. gaucha and the gaucha female x pavani male hybrid larvae used both paper types to pupate (Fig. 4 B, D, Table S2). Notably, more than 70% of the pupae of the four groups of genotypes were located on the agar, and less than 14% were located on the paper types moistened with virgin food and food processed by D. melanogaster larvae (Fig. 4 E–H, Table S2).

Figure 5. A–D shows that D. pavani and the pavani female x gaucha male hybrid larvae that were stimulated by D. melanogaster larval volatiles pupated near congeners (Fig. 5 A, B, black circles). Aggregation decreased in the presence of conspecific larval chemicals (Fig. 5 A, B, white triangles). These differences between the two treatments were statistically important (Table S3). The results confirmed that olfaction is needed for pupation site selection in D. pavani and pavani female x gaucha male hybrid larvae. By contrast, in the presence of conspecific and D. melanogaster larval cues, D. gaucha and gaucha female x pavani male hybrids randomly pupated across the agar (Fig. 5 C, D, white triangles and black circles), which suggested that these larvae did not use olfaction to select their pupation sites.

Stimulated at the same time by conspecific and D. melanogaster larval cues, D. pavani and pavani female x gaucha male hybrid larvae pupated more on conspecific target paper than D. melanogaster paper (Fig. 6 A, B, Table S2). However, a similar percentage of D. gaucha and gaucha female x pavani male hybrid pupae were detected on the two paper types (Fig. 6 C, D, Table S2). Clearly, in the two sibling species divergent evolutionary changes in the organization and function of the larval nervous system involved in pupation behavior have occurred.

Discussion

D. melanogaster and D. pavani third-instar larvae emit, perceive and react to conspecific and alien larval volatiles, influencing pupation behavior. Larvae of the two species puate near congeners and away from pupae of other species. Confronted with D. pavani larval odors, D. melanogaster increased pupa aggregation by decreasing the distance between nearest neighboring conspecific pupae. However, stimulated by their own odors, the distance between pupae increased, thus decreasing the aggregation. D. pavani larvae exhibit behavior comparable to that of D. melanogaster. These plastic features of pupation behavior in the two species are consistent with the fact that they coexist in the
same orchards [15,25,26,35]. D. buzzatii and D. simulans also showed flexible pupation behaviors and increased pupa aggregation in the presence of alien larval odors [14,15]. D. simulans and D. melanogaster belong to the subgenus Sophophora, melanogaster subgroup; D. buzzatii belongs to the subgenus Drosophila, repleta group, buzzatii cluster; and D. pavani, belongs to the subgenus Drosophila, mesophragmatica group [36]. We conclude that olfaction is required for pupation site selection in many Drosophila species.

However, our results also indicated that D. gaucha, the sibling species of D. pavani, did not use larval odor input to select their pupation sites. These behavioral differences are most likely because of the ecological differences between the two sibling species. In Argentina, D. gaucha is reared on cladodes of Opuntia ficus-indica in the absence of D. melanogaster and D. pavani ([24–26], and personal observations). In Chile, D. pavani, D. melanogaster and D. simulans emerge from decaying prickly pear fruits (unpublished data). D. gaucha die when bred on prickly pear fruits. On the other hand, D. melanogaster does not survive on prickly pear cladodes (unpublished data). Thus, in nature, larvae of the two species do not share identical breeding sites. These ecological differences could explain why gaucha larvae do not respond to melanogaster larval volatiles. The deprivation of odor experience may lead to a loss of sensitivity and acuity for distinguishing odorants [37]. We expect that D. melanogaster and D. pavani larvae do not respond to the larval volatiles of D. gaucha. It is also possible that D. gaucha does not emit larval odors.

D. melanogaster

D. melanogaster has a shorter larval period than D. pavani - 4 to 6 days versus 8 to 12 days, at 24°C - [32]. This difference may mean that D. melanogaster larvae searching for pupation sites do not encounter D. pavani larvae and/or pupae. However, this cannot account for why the third-instar larvae of the two species modified their pupation behavior when stimulated by the odors of the other species. Perhaps conspecific and alien larval identification occurs when first and second instar larvae are feeding within the breeding sites. Therefore, it is important to investigate whether first and second instar larvae of Drosophila associate larval odors with the presence of conspecific and alien larvae. Further studies are planned to investigate this possibility.

The chemicals present in the paper used could diffuse through the agar. Thus, larval gustatory responses could be also involved in...
our measurements. Nevertheless, in the presence of conspecific and D. pavani larval odors, the Orco larvae pupated randomly across the agar, and distance between nearest neighboring Orco pupae did not change. These mutant larvae were without the Orco odor co-receptor but had gustatory ones [29]. The Orco mutation interferes with larval odor recognition, thus affecting pupation behavior. These results confirm that olfaction, not gustation, is essential for pupation site selection in D. melanogaster.

On the other hand, stimulated by conspecific odors and D. pavani odor cues, Syn97CS and rut larvae also pupate randomly across the Petri dish agar. Thus, these two mutations cause notable changes in the pupation behavior of D. melanogaster. These two loci affect olfactory associative learning in D. melanogaster [28,30]. We suggest that larval odor-based learning could be involved in the pupation behavior of D. melanogaster, D. buzzatii and D. simulans larvae reared in isolation from conspecifics pupate randomly across the substrate, but form pupa aggregations when reared with congeners [14]. Notably, larvae of Drosophila hydei, subgenus Drosophila, subgenus Drosophila, subgenus Drosophila, subgenus Drosophila and Drosophila busckii and Drosophila also form pupa aggregations away from D. melanogaster and D. pavani pupae [15]. These results suggest that social larval odor-based learning could be an integral component of pupation site selection and it could be widely extended throughout the genus Drosophila.

Larvae of many Drosophila species develop within unpredictable, heterogeneous and rapidly changing breeding environments [8,16–18,35]. Fermentation increases the acidity of fruits, and microbial action also produces alcohols, esters and other odoriferous, toxic substances that cumulate within the breeding sites surrounding the larvae [14,17,18]. On the other hand, decaying fruits are exposed to desiccation reducing their availability as breeding sites. These are all heterogeneous processes because some sections of a fruit may be in an advanced state of dryness and decay, whereas desiccation and fermentation are only beginning in other sections of the fruit. The presence of other species of larvae within the identical decaying fruit unit is another difficult variable to predict. In Chilean orchards three to four Drosophila species may emerge from a fruit, whereas only one species may emerge from the identical fruit type located 15–18 cm away (unpublished data). Thus, many biotic and abiotic variables cause important ecological changes to which Drosophila larvae must respond. In these changing environments, larval odor-based learning could be important to pupation behavior [21]. The spatial positions on the breeding sites where conspecifics emit odor signals could be associated with favorable locations to pupate. Alien larval odors could be indicative of an unfit area colonized by the larvae of other species. Orco, Syn97CS and rut mutations interfere with the association process by causing disturbances and blocking the recognition of odors of congeners and alien larvae. Thus, Orco,
D. melanogaster or D. pavani is phylogenetically close to
olfaction is used for selection of pupation sites. In D. gaucha,
ability to form groups away from alien larvae.

D. pavani and D. gaucha
Notably, when stimulated by D. melanogaster larval cues, D. pavani pupae were grouped, but D. gaucha pupae were observed scattered across the substrate. Thus, D. gaucha larvae show a markedly different behavior to that of D. pavani larvae. Namely, D. gaucha larvae did not appear to use the olfactory system to select their pupation sites. However, these conjectures could be not completely correct. Fig. 4B shows that approximately 33% of D. gaucha pupae were present on the conspecific and sterile food imregnated paper, but in the sterile food/Oregon R-c food treatment, 10% of the pupae were located on the paper (Fig. 4F). These results may be indications that D. gaucha larvae detected D. melanogaster larval volatiles. However, as in nature, D. gaucha was ecologically isolated from D. melanogaster, this result could have occurred because of a delay in processing and transmitting information to central structures. In D. melanogaster, larval olfactory responses may change according to the post-eclosion odor experience [37]. Further experiments will be required to clarify these results.

D.pavani x D.gaucha hybrids
The pupation behavior of the D. pavani x D. gaucha hybrids confirms that the larvae of the two species differed remarkably in their olfactory sensitivity. The pavani female x gaucha male hybrid larvae used olfaction to select their pupation sites, but the gaucha female x pavani male hybrid larvae did not do. However, in the presence of D. melanogaster larval cues, the pavani female x gaucha male hybrid pupae were aggregated, but the gaucha female x pavani male hybrids pupated randomly across the substrate. These results indicate that in D. pavani and D. gaucha genes on the X chromosome may have important behavioral effects.

Alternative alleles appear to be localized on the X chromosome of each species. Reciprocal differences in behavior in crosses between the species could also reflex cytoplasmic heredity. Relatively little attention has been paid to olfactory responses in intra-and interspecific hybrid Drosophila larvae. These investigations may provide key information about the neurogenetics and evolution of behavior in sibling species of Drosophila. Although our experiments demonstrate significant differences in odor responsiveness between D. pavani and D. gaucha, definitive evidence will require an analysis of chemosensory receptors in larvae of the two species.

Our study suggests that in species of Drosophila that coexist in the wild and belong to phylogenetically distant clades, such as D. melanogaster, subgenus Sophophora, and D. pavani, subgenus Drosophila, olfaction is used for selection of pupation sites. In D. gaucha which is phylogenetically close to D. pavani, the larvae do not coexist with D. melanogaster or D. pavani and do not require olfactory signals to select pupation sites. Thus, our results emphasize that the presence of larvae from two or more Drosophila species at breeding sites is significant to understand the role of olfactory system and most likely larval odor-based learning in pupation site selection. Our investigation also emphasizes the significance of genotype, thus providing some information about the genetics of neural circuits underlying to pupation behavior.

In summary, Drosophila larva pupation behavior contributes to the partition of space between species with similar ecologies. This behavior has implications for a stable coexistence of many Drosophila species in nature. Pupation behavior also helps to organize Drosophila communities so that they persist on ephemeral and fragmented breeding sites. The larvae appear to produce some pheromones similar to those observed in adults [16]. In Drosophila, imagos produce cuticular pheromones that chemically correspond to unsaturated long-chain hydrocarbons, and the position of double bonds is key for recognize partners. In D. melanogaster, males produce abundant monoenes, such as 7-tricosene and 7-pentacosene; whereas females produce 7, 11-heptacosadiene and 7, 11-nonacosadiene [38]. Some components of salivary gland secretions might function as pheromones in the larvae. Further studies will be necessary to support this hypothesis.

Supporting Information

Figure S1 Aggregation of pupae observed on cladodes de prickly pear (Opuntia ficus-indica). Pupae correspond to D. buzzatii (repleta group).

Figure S2 Aggregation of pupae observed on grape (Vitis vinifera, Pais variety). Pupae correspond to D. melanogaster (melanogaster subgroup).

Table S1 Chi-square values for differences between replicates within a strain for the indicated species and treatments, degree of freedom = 9. For the all strains of D. melanogaster, the strain x food/Or food interaction (Fig. 3 A-H) yielded Chi-square values near 1.00.

Table S2 Probabilities associated with proportions of pupa on the papers in the indicated treatments (the binomial test). The null hypothesis is no difference between the probability to select one type of paper and the probability to select the other type of paper within a treatment and strain (p = q =½). Probabilities in the Table indicate whether the null hypothesis must be rejected. The null hypothesis (H0) is the pupae are distributed at random over the two papers. When probabilities are smaller than α = 0.01, the decision is to reject H0 in favor of H1, that is, p≥q. For the all strains of D. melanogaster, the strain x food/Or food interaction (Fig. 3 A-H) yielded probabilities greater than α = 0.01, that is p = q =½. For other details, see Table S1.

Table S3 Analysis of variance for pupa aggregation of D. melanogaster expressed as index (R) of aggregation in the presence and in the absence of D. pavani larval volatiles (data in Figure 2). Pupa aggregation of D. pavani, D. gaucha and the hybrids in the presence and in the absence of D. melanogaster larval volatiles are also compared (data in Figure 5). F0.001, 9 values and their probabilities are shown.

Author Contributions

Conceived and designed the experiments: RG-H. Performed the experiments: FDP CJ LP. Analyzed the data: FDP LP RG-H. Contributed reagents/materials/analysis tools: CJ LP. Wrote the paper: RG-H.
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