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TITLE: Evolution of behavioral and cellular defenses against parasitoid wasps in the *Drosophila melanogaster* subgroup

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RUNNING TITLE: Evolution of anti-parasitoid defenses

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ABSTRACT

It may be intuitive to predict that host immune systems will evolve to counter a broad range of potential challenges through simultaneous investment in multiple defenses. However, this would require diversion of resources from other traits, such as growth, survival, and fecundity. Therefore, ecological immunology theory predicts that hosts will

specialize in only a subset of possible defenses. We tested this hypothesis through a comparative study of a cellular immune response and a putative behavioral defense used by eight fruit fly species against two parasitoid wasp species (one generalist and one specialist). Fly larvae can survive infection by melanotically encapsulating wasp eggs and female flies can potentially reduce infection rates in their offspring by laying fewer eggs when wasps are present. The strengths of both defenses varied significantly but were not negatively correlated across our chosen host species; thus, we found no evidence for a trade-off between behavioral and cellular immunity. Instead, cellular defenses were significantly weaker against the generalist wasp, whereas behavioral defenses were similar in strength against both wasps and positively correlated between wasps. We investigated the adaptive significance of wasp-induced oviposition reduction behavior by testing whether wasp-exposed parents produce offspring with stronger cellular defenses, but we found no support for this hypothesis. We further investigated the sensory basis of this behavior by testing mutants deficient in either vision or olfaction, both of which failed to reduce their oviposition rates in the presence of wasps, suggesting that both senses are necessary for detecting and responding to wasps.

KEYWORDS
generalist and specialist parasitoids;
behavioral immunity;
cellular immunity;
life history;
evolutionary trade-offs.

INTRODUCTION

Trade-offs between life history traits such as age vs. size at maturity, quality vs. quantity of offspring, and early vs. late fecundity are commonly observed and well-described (Stearns, 1992). Following the successful tradition of life history theory, studies in the recently established field of ecological immunology have argued that anti-parasite defenses often impose significant costs, leading to reduced investment in other traits such as somatic growth, survival, and fecundity (Boots & Begon, 1993; Sheldon &

Verhulst, 1996; Moret & Schmid-Hempel, 2000; Rolff & Siva-Jothy, 2003; Otti *et al.*, 2012). It is often difficult to explain the underlying mechanisms of these physiological costs (Zera & Harshman, 2001; Rolff & Siva-Jothy, 2003), but autoimmune responses and oxidative stress may be important drivers of reduced lifespan and reproductive potential (Hasselquist & Nilsson, 2012). Trade-offs between immune responses and life history traits can be revealed through artificial selection experiments. For example, fruit fly lineages selected for increased resistance to parasitoid wasps showed reduced competitive ability and reduced feeding rates (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998; 1999b). Given that many defenses are costly, it is expected that hosts have evolved incomplete immune arsenals characterized by investment in some but not all types of immunity (Parker *et al.*, 2011).

Most studies of host-parasite interactions have focused on the molecular basis for cellular and humoral immune mechanisms (Gillespie *et al.*, 1997; Lemaitre & Hoffmann, 2007; Dodds & Rathjen, 2010; Laughton *et al.*, 2011; Parham & Janeway, 2014). However, hosts can also defend themselves against their parasites through behavioral mechanisms. Behavioral immunity was first described in mammals (Janzen, 1978), including great apes, which are believed to use herbal medicines when sick (Wrangham & Nishida, 1983; Huffman & Seifu, 1989; Huffman, 2003). Although it has been argued that big brains are required for such medication behaviors (Sapolsky, 1994), an increasing number of studies indicate that small-brained insects have also evolved a wide variety of anti-parasite behaviors (De Roode & Lefèvre, 2012). For example, gypsy moth larvae prevent infection by avoiding virus-killed moth cadavers (Capinera *et al.*, 1976; Parker *et al.*, 2010), migratory locusts seek out hot temperatures to overcome fungal parasite infection (Inglis *et al.*, 1996), and woolly bear caterpillars increase the intake of anti-parasitoid chemicals in their diet (Singer *et al.*, 2009). Behavioral defenses may not necessarily benefit the individual performing the behavior, but may instead be directed towards genetic kin. For example, parasite-infected monarch butterflies preferentially lay their eggs on milkweeds with high levels of cardenolides, and infected offspring that feed on those plants have reduced spore loads and longer lifespans (Lefèvre *et al.*, 2010; Lefèvre *et al.*, 2012a).

The use of alternative defense mechanisms may render traditional cellular and humoral immune responses superfluous. For example, wood ants that incorporate anti-microbial conifer resin into their nests have reduced investment in humoral antimicrobial activity (Castella *et al.*, 2008a; Castella *et al.*, 2008b). Honey bees have a wide range of behavioral defense mechanisms, whereas the honey bee genome lacks many genes that have immune functions in other insects (Evans *et al.*, 2006). Pea aphids harbor mutualistic bacteria that protect them from endoparasitoid wasps and fungal pathogens (Oliver *et al.*, 2003; Parker *et al.*, 2013), but appear to have lost several canonical insect immune genes (Gerardo *et al.*, 2010). Although these examples are compelling, they do not provide direct evidence for trade-offs between alternative defenses across species. Here, we address this outstanding question by comparing the relative strengths of cellular and behavioral immune responses mounted by eight fruit fly species against two parasitoid wasp species.

Fruit flies in the *Drosophila melanogaster* subgroup (Lachaise *et al.*, 1988; David *et al.*, 2007) likely coevolved with endoparasitoid wasps in the genus *Leptopilina* (Hymenoptera: Cynipoidea, Figitidae) across their ancestral African ranges (Allemand *et al.*, 2002). These wasps lay eggs in fly larvae and can impose strong selective pressure on fly populations, given that natural rates of parasitism can exceed 90% (Fleury *et al.*, 2004). Female wasps probe fly larvae with their ovipositors and inject venom along with an egg once they find a suitable host. Fly larvae that have been attacked can activate a cellular immune response known as melanotic encapsulation, in which the wasp egg is recognized as foreign, activated plasmatocytes bind to it, and lamellocyte production is induced in the lymph gland. After plasmatocytes and lamellocytes have formed a multilayered capsule around the wasp egg, cells inside the capsule release free radicals and the melanin-generating enzyme phenoloxidase, and the developing wasp presumably dies due to toxicity, asphyxiation, or physical entrapment. Wasps have evolved a wide range of counter-defenses; for example, eggs can avoid complete encapsulation by attaching to host tissues and venom can cause host lamellocytes to lose adhesiveness or lyse. Interactions between immune activation and suppression largely determine whether the host or the parasitoid survives to adulthood. Adult flies

that have survived parasitism carry melanized capsules that can be observed through the abdominal cuticle or by dissecting the fly (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Carton & Nappi, 1997; Lemaitre & Hoffmann, 2007; Carton *et al.*, 2008; Keebaugh & Schlenke, 2014).

Female flies practice oviposition behaviors that may help protect their offspring from wasps, potentially serving as alternatives or complements to larval melanotic encapsulation responses. Lefèvre *et al.* (2012b) found that both *D. melanogaster* and *D. simulans* preferred to lay eggs at clean sites versus wasp-infested sites, which likely reduces the risk that their offspring will be parasitized. This behavior appears to be driven by olfactory cues, as Ebrahim *et al.* (2015) found that *D. melanogaster* avoided oviposition sites perfumed with wasp odors. In addition to the preference for wasp-free oviposition sites, Lefèvre *et al.* (2012b) showed that female *D. melanogaster* laid significantly fewer eggs when they were forced to live in vials with wasps. We measured melanotic encapsulation (cellular immunity) and oviposition reduction (behavioral avoidance) responses mounted by seven of the nine species of the *D. melanogaster* subgroup, plus the outgroup species *D. suzukii*, against two wasp species, the specialist *Leptopilina boulardi* and the generalist *L. heterotoma* (Carton *et al.*, 1986; Schlenke *et al.*, 2007). We tested for immune system trade-offs by assessing correlations between cellular immunity and behavioral avoidance across fly species in response to both wasps. Using the same dataset, we analyzed both responses separately to determine whether immunity against the specialist wasp was a significant predictor of immunity against the generalist wasp.

Reduced oviposition in the forced presence of wasps may be related to preference for wasp-free sites in choice tests (Lefèvre *et al.*, 2012b; Ebrahim *et al.*, 2015), or may be adaptive through an alternative mechanism. Here, we tested one such adaptive explanation, which is that flies are subject to a trade-off between offspring quality and quantity (Stearns, 1992). Specifically, female flies that respond to wasp exposure by producing fewer offspring may produce higher-quality offspring that have enhanced immunity against parasitoid wasps (Lefèvre *et al.*, 2012b). We tested this offspring

quality vs. quantity trade-off hypothesis by comparing the melanotic encapsulation responses of *D. yakuba* offspring derived from control and wasp-exposed parents.

We further investigated visual and olfactory cues as possible triggers of oviposition reduction behavior, using vision- and olfaction-deficient *D. melanogaster* strains. It was recently reported that female *D. melanogaster* use the olfactory receptors Or49a and Or85f to detect *L. boulardi* odors, including the sex pheromone iridomyrmecin, and strongly avoid laying eggs at sites with those odors (Ebrahim *et al.*, 2015). Visual detection of wasps can trigger reduced neuropeptide F signaling in the fan-shaped body of fly brains (Kacsoh *et al.*, 2013), which might also lead to changes in oviposition behavior.

MATERIALS AND METHODS

Insect strains and maintenance

The *D. melanogaster* subgroup consists of nine species that originated in Africa: *D. erecta*, *D. orena*, *D. mauritiana*, *D. melanogaster*, *D. santomea*, *D. sechellia*, *D. simulans*, *D. teissieri*, and *D. yakuba* (Lachaise *et al.*, 1988; David *et al.*, 2007). We did not use *D. mauritiana* or *D. sechellia* in this study, but added *D. suzukii* as an outgroup because it differs from the other fly species in ecological niche, evolutionary history, and immune traits. *D. suzukii* is an invasive fresh fruit pest that originated in east Asia and has recently spread to Europe and North America (Adrion *et al.*, 2014; Atallah *et al.*, 2014). *D. suzukii* larvae have high constitutive hemocyte loads and mount successful melanotic encapsulation responses against a broad range of wasps that successfully parasitize *D. melanogaster* (Kacsoh & Schlenke, 2012; Poyet *et al.*, 2013).

D. erecta (strain 14021-0224.01), *D. orena* (strain 14021-0245.01), *D. santomea* (strain 14021-0271.00), *D. teissieri* (strain 14021-0257.01), and *D. yakuba* (strain 14021-0261.01) were acquired from the Drosophila Species Stock Center at UC San Diego. Multiple lines of *D. melanogaster*, *D. simulans*, and *D. suzukii* were established from single females collected in Atlanta, GA in 2013 using rotting fruit traps. Ten *D. melanogaster*, ten *D. simulans*, and three *D. suzukii* isofemale lines were interbred to

create genetically variable populations of each species. Four *D. melanogaster* strains were acquired from the Bloomington Drosophila Stock Center: the wild-type strains *Canton S* (strain 1) and *Oregon R* (strain 5), the white-eyed mutant w^{1118} (strain 5905), and the olfaction-deficient mutant $Orco^{2}$ (strain 23130). *Orco* is a broadly expressed odorant receptor that interacts with specialized receptors to mediate responses to a diverse range of olfactory stimuli. Insertion of the wild-type eye color marker *mini-white* into the *Orco* coding region complements the *white*-null background and leads to defective larval and adult olfactory responses in the null mutant $Orco^{2}$ (Larsson *et al.*, 2004; Vosshall & Hansson, 2011). The sight-deficient *D. melanogaster* strain GMR-*hid* was kindly provided by K.H. Moberg. Ectopic expression of the *head involution defective* (*hid*) gene under the control of GMR, an eye-specific promoter, causes cell death in the developing retina and results in ablated eyes (Grether *et al.*, 1995).

The *Drosophila* medium used for all experiments and maintenance of fly and wasp stocks was prepared by adding 20 L cold water, 1480 g yellow cornmeal (Fisher), 640 g inactive dry yeast (Genesee Scientific), 200 g *Drosophila* agar (Genesee Scientific), and 1750 mL molasses (Good Food, Inc.) to a steam kettle on the “high” stir setting. The food was simmered and stirred for 30 minutes, then cold water was added to a total volume of 32 L, followed by 460 g Tegosept mold inhibitor (Genesee Scientific) and 100 mL propionic acid (Fisher). After an additional 5-10 minutes of cooking, the food was dispensed into vials or bottles. This recipe makes 20 trays of 100 wide vials or 25 square-bottom bottles (Genesee Scientific).

We used two larval endoparasitoid wasp species in the genus *Leptopilina* (Hymenoptera: Cynipoidea, Figitidae) for all experiments: *L. boulardi* (strain Lb17) and *L. heterotoma* (strain Lh14) are inbred strains generated from single females collected in Winters, CA in 2002 (Schlenke *et al.*, 2007). *L. boulardi* and *L. heterotoma* have different host ranges and virulence strategies, leading us to expect substantial variation in the strengths of behavioral and cellular defenses across fly species. *L. boulardi* is a more specialized wasp that often parasitizes flies in the *D. melanogaster* / *D. simulans* clade in nature, whereas *L. heterotoma* is a generalist wasp that successfully

parasitizes diverse species across the genus *Drosophila* (Carton *et al.*, 1986; Fleury *et al.*, 2004). Differences in the immune-suppressive effects of *L. boulardi* and *L. heterotoma* venom in *D. melanogaster* larvae may partially explain this difference in host range (Schlenke *et al.*, 2007). *L. heterotoma* venom directly attacks circulating host lamellocytes, causing morphological changes, loss of adhesiveness, and eventually lysis (Rizki & Rizki, 1984). *L. boulardi* venom can alter host lamellocyte morphology to some extent, but does not lyse lamellocytes, and *L. boulardi* eggs can evade complete encapsulation by attaching to host tissues (Rizki *et al.*, 1990). Wasps were maintained by allowing *D. melanogaster* strain *Canton S* flies to lay eggs in food vials for 3 days, then removing the flies and adding ~10 mated female wasps. Adult wasps were kept at 18□ in food vials with 1/2 rolled Kimwipe pushed into the center of the food (to control the humidity) and the cotton plug supplemented with 50% honey water (to provide a food source). Wasps were aged 3–7 days before using them in experiments. All experiments were conducted in a 25□ incubator with a 12 hr light : 12 hr dark cycle.

Cellular immunity assays

Bottles with 50 mL *Drosophila* medium and 10 mL water were microwaved to liquefy the food. 35 mm and 60 mm Petri dishes were filled approximately halfway with liquefied food and cooled at 4□. Large groups of flies (> 200 adults) were placed in cylindrical mesh-topped embryo collection chambers (Genesee Scientific #59-100) with 60 mm food dishes and allowed to lay eggs. The food dishes were replaced every 2 days until sufficient numbers of fly larvae had been collected. Before each assay, groups of 40–60 female wasps were placed on *Oregon R* egglay dishes with first and second-instar larvae and allowed to attack for 2 hrs. We expected these “experienced” wasps to attack more efficiently in the future. Sets of 50 second-instar fly larvae were transferred from egglay dishes to 35 mm food dishes and exposed to 6 experienced female wasps (Lb17 or Lh14) for 3 hrs. Two days later, 10 larvae from each dish were dissected. Replicates in which fewer than 7 of those 10 larvae contained wasp eggs, wasp larvae, or melanized capsules (i.e. wasp attack rate was <70%) were discarded from the statistical analyses. The remaining larvae were counted and transferred to food vials with 1/2 rolled Kimwipe in the center of the food (to provide a pupation surface). These

vials were checked daily for eclosed flies and wasps for approximately 5 weeks. Eclosed flies were visually examined or dissected to check for melanized capsules, which indicate that the fly survived parasitism. Flies without capsules were considered unattacked and ignored in the analyses. All transferred fly larvae that did not eclose were assumed to have died from wasp attacks. Based on the possible eclosion outcomes for attacked larvae: (i) fly survival, (ii) wasp survival, and (iii) death, cellular immunity indices [i / (i + ii + iii)] and proportional eclosion outcomes were calculated for each dish replicate, then averaged across replicates. The number of dish replicates, total eclosion outcomes, and mean cellular immunity index for each fly-wasp combination are shown in Table S1.

Forced co-habitation assays

Flies were allowed to eclose for 3 days, then collected in food vials, aged for 24 hrs, sorted into groups of 25 female and 5 male flies, and given 24 hrs to recover from CO_2 anesthesia. The resulting groups of 2–5 day old flies were transferred to new vials in 3 treatments: (i) no wasps (control), (ii) 8 female Lb17, or (iii) 8 female Lh14. Insects were transferred to new vials every 24 hrs for 5 days, using very light anesthesia to avoid losing insects and minimize any possible effects of CO_2 on behavior and mating success (Barron, 2000). Dead female flies and eggs laid in each vial were counted daily, then cumulative per-female egg counts over 5 days (PFEC) were calculated. Ideally, 5 replicates per treatment (control, Lb17, and Lh14) were performed simultaneously. However, for some fly species it was impossible to obtain 375 3–5 day old females (15 vials x 25 females per vial) in a single generation, so multiple experiments were conducted with 2–4 replicates per treatment. Within each experiment, oviposition maintenance indices for each Lb17 and Lh14 replicate were calculated as $[PFEC_{Lb17} / \text{mean}(PFEC_{control})]$ or $[PFEC_{Lh14} / \text{mean}(PFEC_{control})]$ and considered independent replicates. The number of vial replicates, mean PFEC for each treatment, and mean oviposition maintenance index for each fly-wasp combination are shown in Table S2.

Adaptive significance of behavioral avoidance

Groups of 25 female and 5 male *D. yakuba* aged 1–3 days were placed in vials to generate 3 parental treatments: (i) no wasps, (ii) 10 female Lb17, or (iii) 10 female Lh14. After a 3-day acclimation period, the insects were transferred to new vials daily for 6 days. Eggs were counted every 24 hrs and cumulative per-female egg counts were calculated. On days 3 and 4, fine forceps were used to transfer unhatched eggs from the vials into 35 mm food dishes. Due to variation in oviposition and hatching rates, the number of eggs per dish ranged from 20–44. Collecting unhatched eggs was necessary because some fly larvae that hatched in the egglay vials had already been attacked by wasps, making them unsuitable for controlled wasp exposures. 60 hrs later, when most larvae had grown to second instar, 6 female Lb17 were added to each dish for 3-hr exposures. We focused on *D. yakuba* and Lb17 because this host-parasitoid combination resulted in substantial fly survival, wasp survival, and death in our cellular immunity assays (Table S1), suggesting that wasp-induced changes in parental investment might lead to enhanced offspring immune responses and higher fly survival. Fly larvae were not exposed to Lh14 because baseline resistance to Lh14 is extremely low (Table S1) and seems unlikely to change with different parental treatments. Immediately following the wasp exposures, the contents of each dish (food and larvae) were scooped into food vials with 1/2 rolled Kimwipe. Approximately 2 weeks later, surviving fly offspring with and without melanized capsules were counted. We assumed that fly larvae could survive exposure to Lb17 by mounting a successful cellular immune response (adults with capsules) or avoiding parasitism (adults without capsules), and both outcomes were compared across parental treatments. A similar experiment was performed with *D. melanogaster* and *D. simulans* (Supporting Information).

Sensory basis of behavioral avoidance

The behavioral responses of wild-type (*Oregon R*), white-eyed mutant (w^{1118}), sight-deficient mutant (GMR-*hid*), and olfaction-deficient mutant ($Orco^{2}$) *D. melanogaster* strains to Lb17 and Lh14 were measured using the previously described forced co-habitation assays. We hypothesized that the wild-type flies would reduce their oviposition rates in the presence of wasps and that one or more mutants would fail to respond, suggesting that detection of wasps requires vision, olfaction, or both.

Phylogenetic analysis

We were unable to find a published phylogeny with all eight of our chosen fly species to use for calculating phylogenetically independent contrasts. Therefore, we constructed a phylogeny using the amylase-related protein gene *Amyrel* (Da Lage *et al.*, 1998), which has been used to estimate multiple *Drosophila* phylogenies (Cariou *et al.*, 2001; Kopp, 2006; Da Lage *et al.*, 2007; Yang *et al.*, 2012). Although we were unable to find any gene in GenBank with complete coding sequences for all eight fly species, we found a partial *Amyrel* coding sequence for *D. suzukii* and complete coding sequences for the other seven species (Table S3). These sequences were aligned and trimmed in Mesquite version 2.75 (Maddison & Maddison, 2011) using MUSCLE version 3.8.31 (Edgar, 2004), resulting in an aligned region of 1411 base pairs. The fly phylogeny was estimated using MrBayes version 3.2 (Ronquist *et al.*, 2012), which implements Bayesian inference (Larget & Simon, 1999) within a Markov Chain Monte Carlo simulation framework (Metropolis *et al.*, 1953; Hastings, 1970). Nucleotide substitution rate parameters were estimated using a generalized time-reversible model (Rodriguez *et al.*, 1990) with gamma-distributed rate variation across sites (Yang, 1993). Two separate runs with four interacting chains were executed for 1,000,000 generations and sampled every 500 generations. The first 25% of the posterior distributions were regarded as burn-in and ignored. The resulting 50% majority rule consensus tree (Fig. 1) was graphically prepared using FigTree version 1.4.2 (Rambaut, 2014). The topology of the tree is consistent with previous studies (Da Lage *et al.*, 2007; Yang *et al.*, 2012).

Statistical analysis

Unless otherwise stated, all analyses were performed in R version 3.0.3 (R Development Core Team, 2014). For cellular immunity data, the effects of fly species, wasp species, and their interaction on proportional fly survival were analyzed using generalized linear models (GLMs) with quasi-binomial error distributions and logit link functions. Pairwise comparisons between fly species' responses to *L. boulardi* were assessed using Tukey's honestly significant difference (HSD) tests. *D. melanogaster*, which never survived parasitism by either wasp (Fig. 2), was removed from the overall analysis and manually assigned to the lowest significance group because the logistic

regression algorithm does not converge when proportional fly survival equals zero. Pairwise comparisons were not carried out for responses to *L. heterotoma* because only *D. suzukii* had an appreciable survival rate. All pairwise comparisons were assessed using the R package multcomp (Hothorn *et al.*, 2008).

For behavioral avoidance data, the effects of fly species, wasp species, and their interaction on log-transformed oviposition reduction indices were analyzed using an ANOVA. Shapiro-Wilk normality tests ($W > 0.843$, $P > 0.062$ for all combinations of fly and wasp species), visual inspection of the normal Q-Q plot for the entire dataset, and a Fligner-Killeen test comparing variances across fly-wasp combinations ($\chi^2_{15} = 21.61$, $P = 0.119$) suggested that the log-transformed oviposition reduction indices did not violate the normality or homoscedasticity assumptions of ANOVA. The effects of wasp treatment (control, Lb17, or Lh14) on cumulative per-female egg counts over 5 days were analyzed individually for each fly species using GLMs with quasi-Poisson error distributions and log link functions, then pairwise comparisons were assessed using Tukey's HSD tests.

Traits measured across species cannot be considered statistically independent because evolutionary changes are shared along internal branches of phylogenetic trees. We addressed this problem by calculating phylogenetically independent contrasts (Felsenstein, 1985) from the mean cellular immunity and oviposition maintenance indices for each fly-wasp combination using the *Amyrel* phylogeny (Fig. 1) and the Contrast program in PHYLIP version 3.69 (Felsenstein, 2005). Correlations between oviposition maintenance vs. cellular immunity index contrasts measured across fly species in response to (i) *L. boulardi* and (ii) *L. heterotoma* were assessed to test for immune system trade-offs. Significant positive correlations would support the trade-off hypothesis because high oviposition maintenance indices indicate weak behavioral avoidance. Correlations between (i) cellular immunity index contrasts and (ii) oviposition maintenance index contrasts measured across fly species in response to *L. heterotoma* vs. *L. boulardi* were assessed to determine whether defenses against the specialist wasp were significant predictors of defenses against the generalist wasp. Felsenstein's

(1985) method produces pairs of contrasts that can be regarded as being drawn from a bivariate normal distribution with mean = 0 and variance = 1. Therefore, a Pearson product-moment correlation coefficient test was performed in each case, with the null hypothesis that the correlation between paired contrasts was equal to 0. Transforming the branch lengths of the phylogeny based on: (i) equal branch lengths, (ii) Grafen's (1989) method, and (iii) Pagel's (1992) method, implemented in version 1.15 of the PDAP:PDTREE package for Mesquite (Midford *et al.*, 2009), did not qualitatively change our conclusions. Only the results from the PHYLIP analysis based on our *Amyrel* phylogeny are reported.

For the *D. yakuba* adaptive significance experiment, the effects of parental treatment (control, Lb17-exposed, or Lh14-exposed) on cumulative per-female egg counts over 6 days were analyzed using GLMs with quasi-Poisson error distributions and log link functions, followed by pairwise comparisons using Tukey's HSD tests. The effects of parental treatment on proportional offspring survival following exposure to Lb17 were analyzed using GLMs with quasi-binomial error distributions and logit link functions, followed by pairwise comparisons using Tukey's HSD tests. Separate analyses were performed for the two survival outcomes (with or without a melanized capsule) and their sum. To evaluate the sensory basis of behavioral avoidance, the effects of wasp treatment (control, Lb17, or Lh14) on cumulative per-female egg counts over 5 days were analyzed using GLMs with quasi-Poisson error distributions and log link functions, followed by pairwise comparisons using Tukey's HSD tests. Separate analyses were performed for each fly strain.

RESULTS

Fly larvae can survive wasp infection by melanotically encapsulating wasp eggs and female flies can potentially reduce infection rates in their offspring by laying fewer eggs when wasps are present. To test for trade-offs between these defenses and assess their generality across host species, we measured cellular immune responses and oviposition reduction behaviors using eight fly species and two wasp species. We defined two summary statistics: (i) cellular immunity index, the mean proportional fly

survival following wasp attack; and (ii) oviposition maintenance index, the cumulative per-female egg count during 5-day assays (PFEC) for females that were forced to live with wasps divided by PFEC for females kept in wasp-free environments.

Cellular immunity indices (Fig. 2, equivalent to proportional fly survival) varied significantly across fly species ($F_{6,138} = 89.5$, $P < 0.0001$); resistance to *L. boulardi* was stronger overall ($F_{1,137} = 468$, $P < 0.0001$), and there was a significant interaction between fly species and wasp species ($F_{6,131} = 34.6$, $P < 0.0001$). *D. santomea* and *D. suzukii* had the strongest resistance to *L. boulardi*, followed by *D. teissieri*, *D. yakuba*, *D. orena*, and *D. erecta*, whereas *D. simulans* had very weak resistance and *D. melanogaster* never survived parasitism (Fig. 2a; $z > 3.78$, $P < 0.003$ for all significant pairwise comparisons). Most fly species had no resistance to *L. heterotoma* (Fig. 2b). However, *D. suzukii* was an unsuitable host for both wasps, surviving parasitism approximately 60% of the time and never allowing either wasp to develop successfully.

Oviposition maintenance indices (Fig. 3) varied significantly across fly species ($F_{7,92} = 14.7$, $P < 0.0001$), but there was no effect of wasp species ($F_{1,92} = 0.046$, $P = 0.831$) and no significant interaction between fly species and wasp species ($F_{7,92} = 1.31$, $P = 0.256$). Most fly species responded to both wasps by significantly reducing their oviposition rates. Specifically, *D. erecta* and *D. orena* maintained similar oviposition rates regardless of wasp treatment ($z < 1.04$, $P > 0.554$ for control vs. *L. boulardi* and control vs. *L. heterotoma* comparisons), whereas all other fly species significantly reduced their oviposition rates in response to both wasps ($z > 2.32$, $P < 0.05$ for control vs. *L. boulardi* and control vs. *L. heterotoma* comparisons).

Cellular immunity and behavioral avoidance may carry significant costs (Kraaijeveld *et al.*, 2002; Lefèvre *et al.*, 2012b) and both traits varied significantly across fly species, suggesting that trade-offs might occur between these defenses. We tested for trade-offs using phylogenetically independent contrasts to control for shared evolutionary history and non-independence of traits across fly species (Felsenstein, 1985). We found no significant correlations between cellular immunity and behavioral avoidance responses

measured across fly species in response to *L. boulardi* (Fig. 4a; $r = 0.510$, $t_5 = 1.33$, $P = 0.242$) or *L. heterotoma* (Fig. 4b; $r = –0.329$, $t_5 = –0.778$, $P = 0.472$), indicating that these defenses do not trade off across the fly phylogeny.

L. heterotoma can develop successfully on multiple hosts across the genus *Drosophila*, whereas *L. boulardi* has a more restricted host range that includes the *D. melanogaster* group (Carton *et al.*, 1986; Schlenke *et al.*, 2007). This difference in host range might be explained by differences in immune suppression mechanisms (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Schlenke *et al.*, 2007), leading us to predict that fly cellular immune responses would not necessarily be effective against both wasp species. The strengths of cellular immune responses against *L. heterotoma* vs. *L. boulardi* were not positively correlated across fly species (Fig. 4c; $r = 0.270$, $t_5 = 0.628$, $P = 0.558$), suggesting that resistance to the specialist wasp is a poor predictor of resistance to the generalist wasp. However, the strengths of behavioral avoidance responses to *L. heterotoma* vs. *L. boulardi* were positively correlated across fly species (Fig. 4d; $r = 0.944$, $t_5 = 6.41$, $P = 0.00137$), suggesting that female flies do not distinguish between these wasp species when reducing oviposition.

Laying fewer eggs in the presence of wasps could be an adaptive behavior if the resulting offspring have enhanced immunity against wasps (Lefèvre *et al.*, 2012b). We tested this hypothetical offspring quality vs. quantity trade-off (Stearns, 1992) by measuring the oviposition rates of *D. yakuba* parents in three treatments: (i) control (no wasps), (ii) exposed to *L. boulardi*, and (iii) exposed to *L. heterotoma*, then measuring offspring survival after exposure to *L. boulardi*. Female flies that were exposed to wasps laid significantly fewer eggs over 6 days than control parents (Fig. 5a; $z > 3.10$, $P < 0.006$ for control vs. Lb17 and control vs. Lh14 comparisons). There was a significant effect of parental treatment on offspring resistance to *L. boulardi* (Fig. 5b; $F_{2,16} = 4.24$, $P = 0.0332$). Offspring of control and Lb17-exposed parents had similar melanotic encapsulation success against Lb17 ($z = 0.202$, $P = 0.978$), whereas offspring of Lh14-exposed parents had significantly lower encapsulation success ($z = 2.65$, $P = 0.0214$). The majority of surviving offspring of Lh14-exposed parents lacked melanized capsules,

indicating that they avoided parasitism. However, there was no significant effect of parental treatment on the proportion of unparasitized offspring (Fig. 5b; $F_{2,16} = 3.31$, $P = 0.0628$) or total offspring survival (Fig. 5b; $F_{2,16} = 0.434$, $P = 0.655$). Wasp-exposed parents laid significantly fewer eggs and their offspring did not have enhanced survival when exposed to wasps, suggesting that this behavior is costly to fly fitness. Thus, we found no evidence for a trade-off between offspring quality and quantity.

To investigate the sensory basis of oviposition reduction, we compared the behaviors of wild-type and white-eyed *D. melanogaster* to those of vision- and olfaction-deficient mutants. Both wild-type (*Oregon R*) and white-eyed (w^{1118}) flies had significantly reduced cumulative per-female oviposition over 5 days when they were forced to live with female wasps (Fig. 6a,b; $z > 3.44$, $P < 0.002$ for control vs. Lb17 and control vs. Lh14 comparisons). In contrast, the sight-deficient mutant GMR-*hid* and the olfaction-deficient mutant $Orco^2$ showed no significant changes in oviposition when housed with either wasp (Fig. 6c,d; $z < 1.76$, $P > 0.18$ for control vs. Lb17 and control vs. Lh14 comparisons). These results suggest that female *D. melanogaster* require both visual and olfactory cues to detect parasitoid wasps and reduce their oviposition rates.

DISCUSSION

We measured melanotic encapsulation and oviposition reduction responses across all combinations of eight fruit fly species and two larval endoparasitoid wasp species (Figs. 2 and 3). We found no significant correlations between the strengths of these defenses and thus no evidence that they trade off across fly species (Fig. 4a,b). Resistance to *L. boulardi* was a poor predictor of resistance to *L. heterotoma* (Fig. 4c). This is not surprising because the two wasps have different virulence mechanisms, *L. heterotoma* has a broader natural host range, and we expected *L. heterotoma* to be more virulent across our chosen host species (Rizki & Rizki, 1984; Carton *et al.*, 1986; Rizki *et al.*, 1990; Schlenke *et al.*, 2007). Oviposition reduction responses to both wasps were positively correlated across fly species (Fig. 4d), suggesting that this behavior is generalized, not attuned to different wasp species based on their virulence levels.

Previous studies using fruit flies and parasitoid wasps have revealed trade-offs between cellular immune responses and life history traits. *D. melanogaster* lines artificially selected for increased resistance to *Asobara tabida* and *L. boulardi* evolved approximately 11-fold and 100-fold increases in melanotic encapsulation ability (respectively) within just five generations, but showed reduced competitive success in parasite-free environments with severely limited food (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998). Subsequent studies identified physiological changes underlying this trade-off: larvae from the selected lines had increased circulating hemocyte densities (Kraaijeveld *et al.*, 2001) and reduced feeding rates (Fellowes *et al.*, 1999b). Lefèvre et al. (2012b) tested for a possible trade-off within fly immune systems by assaying behavioral and cellular immune responses mounted by the sister species *D. melanogaster* and *D. simulans* against *L. boulardi*. Females of both species avoided laying eggs in wasp-infested oviposition sites when given a choice, but only *D. melanogaster* laid fewer eggs when forced to live with wasps. Conversely, a substantial proportion of *D. simulans* larvae successfully encapsulated wasp eggs, whereas no *D. melanogaster* larvae survived parasitism, suggesting that *D. simulans* invests more resources in cellular immunity than behavioral defense. We expanded the Lefèvre et al. (2012b) study by testing a broader range of host-parasitoid interactions but failed to confirm any consistent trade-off between melanotic encapsulation and oviposition reduction (Fig. 4a,b).

The lack of a trade-off was surprising given that the strengths of both defenses varied significantly across fly species (Figs. 2 and 3). We would expect this variation to manifest as a negative correlation between the two defenses across host species, assuming that both traits involve fitness costs and benefits. However, there are several reasons why this might not have occurred. Perhaps simultaneous investment in both strategies is favored because they are only partially successful when used alone. Another possibility is that one or both defenses do not entail a significant cost. Encapsulation success is positively correlated with level of constitutive hemocyte production (Eslin & Prevost, 1998; Kraaijeveld *et al.*, 2001; Sorrentino *et al.*, 2004; Moreau *et al.*, 2005), which is likely to require substantial resource investment. If larvae

delay pupation and continue eating until they have reached a threshold weight (Robertson, 1963), then the physiological costs to larvae of mounting an encapsulation response may be disconnected from adult fitness. However, fly larvae that successfully defend themselves against wasps may suffer significant fitness costs as adults, including smaller body size and reduced fecundity (Fellowes *et al.*, 1999a), and weaker resistance to desiccation and starvation stresses (Hoang, 2001).

The immediate fitness cost of laying fewer eggs in the presence of wasps is obvious, but there are at least three ways in which this behavior could be adaptive. First, flies may withhold eggs where wasps are present, then lay them in non-infested oviposition sites in the future. In previous work, flies continued to lay fewer eggs even after they were moved to new vials without wasps (Lefèvre *et al.*, 2012b). However, delayed reproduction may occur in natural situations where potential associations between environment and wasp presence are not as strong as in lab vials. Second, female flies might modulate their allocation of reproductive resources to produce fewer offspring with enhanced immunity. However, we found that *D. yakuba* housed in wasp-infested environments produced fewer offspring and those offspring were not better able to survive exposure to *L. boulardi* (Fig. 5). We obtained similar results when we tested *D. melanogaster* and *D. simulans* against both wasp species (Supporting Information, Fig. S1). Third, wasp-exposed parents could produce offspring that are better able to avoid wasp attacks. Examples of larval avoidance include rolling towards the attacking wasp to disrupt ovipositor penetration (Hwang *et al.*, 2007) and crawling away from wasp semiochemicals (Ebrahim *et al.*, 2015). Unparasitized offspring will not suffer the costs of mounting anti-wasp immune responses, which can include smaller body size, reduced fecundity, and weaker stress resistance (Fellowes *et al.*, 1999a; Hoang, 2001). Future studies should compare offspring derived from control and wasp-exposed parents by measuring avoidance mechanisms in larvae and life history traits in adults that survive exposure to wasps.

We must also consider the possibility that the wasp-mediated oviposition reduction behavior is a byproduct of some other adaptive behavior and is not adaptive itself. Insects that do not exhibit parental care must lay their eggs in food sources that can reliably support offspring development, and we expect females to avoid unsuitable oviposition sites if nearby alternatives are available (Jaenike, 1978). For example, Lefèvre et al. (2012b) found that female *D. melanogaster* and *D. simulans* avoided wasp-infested sites during oviposition preference experiments. Although oviposition reduction behavior might appear to be a maladaptive stress response in the context of artificial forced co-habitation experiments, perhaps this behavior manifests as adaptive avoidance of wasp-infested sites when multiple choices are available. Future studies should investigate this possibility by conducting oviposition choice experiments (rather than the forced co-habitation assays used here). Measuring wasp avoidance behavior in this way might lead to different conclusions about trade-offs with cellular immunity across fly species. Before testing for trade-offs with other defenses, it would be ideal to characterize the costs and benefits of parasite-induced changes in oviposition behavior. For example, the seed beetle *Mimosestes amicus* can lay inviable eggs as "shields" to protect the eggs below from the parasitoid wasp *Uscana semifumipennis* (Deas & Hunter, 2012). However, *M. amicus* detects parasitized eggs on potential host plants and may choose to delay oviposition or seek a clean site rather than suffer the costs of laying defensive eggs (Deas & Hunter, 2013).

Olfactory cues trigger a diverse range of behavioral responses in fruit flies and their natural enemies. *D. melanogaster* avoids oviposition sites containing toxic microbes that produce the volatile compound geosmin, which is detected by the odorant receptor *Or56a* (Stensmyr *et al.*, 2012). Conversely, *D. melanogaster* selectively oviposits on *Citrus* spp. fruits, which produce volatile terpenes that activate the odorant receptor *Or19a*. Valencene, the primary ligand of *Or19a*, strongly repels *L. boulardi*, and fly larvae feeding on valencene-containing substrates experience reduced rates of parasitism (Dweck *et al.*, 2013). *L. boulardi* and *L. heterotoma* have evolved foraging behaviors that exploit host odorants, such as water-soluble kairomones produced by larvae and aggregation pheromones deposited by female flies during oviposition (Vet *et*

al., 1993; Wiskerke *et al.*, 1993; Hedlund *et al.*, 1996). However, the possible roles of visual cues in fly-parasite interactions are not as well understood. Our present study suggests that some behavioral responses to parasitoid wasps require interpretation of both olfactory and visual cues. Wild-type *D. melanogaster* strains significantly reduced their oviposition rates in the presence of wasps, whereas mutants deficient in either olfaction or vision failed to show this response (Fig. 6).

There is one potential caveat with our result: the olfaction-deficient mutant $Orco^2$ was created by inserting a *mini-white* gene into the *Orco* coding region in a *white*-null background (Larsson *et al.*, 2004). Various behavioral deficiencies have been reported in *white* mutants, including impaired optomotor responses (Kalmus, 1943), reduced anesthetic sensitivity (Campbell & Nash, 2001), and enhanced male-male courtship (Anaka *et al.*, 2008; Krstic *et al.*, 2013). Furthermore, insertion of *mini-white* into *white*-null backgrounds does not always restore wild-type eye coloration (Hazelrigg *et al.*, 1984; Silicheva *et al.*, 2010) or behavior (Krstic *et al.*, 2013). We observed a range of eye coloration in $Orco^2$ flies, including pale red, orange, and slightly lighter than wild-type red. However, to address these concerns, we tested the *white*-null strain w^{1118}. Any deficiencies associated with *white*-null mutants should be fully expressed in w^{1118} and may be partially rescued in $Orco^2$. We found that w^{1118} showed the same wasp-induced oviposition reduction behavior as the wild-type *Oregon R*, suggesting that the absence of this behavior in $Orco^2$ is most readily explained by olfaction deficiency, not genetic background effects (Fig. 6).

Given the benefits of anti-parasite defenses, an intuitive expectation is that hosts should simultaneously maximize their investment in a wide range of defenses. However, the emerging view from the recently established field of ecological immunology is that defenses are costly and we should expect trade-offs between alternative immune responses (Sheldon & Verhulst, 1996; Norris & Evans, 2000; Rolff & Siva-Jothy, 2003; Ardia *et al.*, 2011; Parker *et al.*, 2011). Despite substantial evidence that trade-offs occur within the immune systems of individual species, comparative studies of immune arsenals used by multiple host species against their shared parasites are lacking. The

Drosophila-Leptopilina system appears to be an excellent model for studying trade-offs between different immune mechanisms. However, we found no evidence for such a trade-off between melanotic encapsulation and oviposition reduction. Future studies incorporating multiple immune mechanisms from each host life stage and/or different measures of behavioral avoidance might be required to validate this key prediction of ecological immunology theory.

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FIGURE LEGENDS

Fig. 1: Phylogeny of the eight fly species constructed from *Amyrel* coding sequences using Bayesian inference. Estimated branch lengths are shown and posterior probabilities are given for each node.

Fig. 2: Mean proportions of fly larvae attacked by (a) *L. boulardi* and (b) *L. heterotoma* that: (i) eclosed as flies with melanized capsules, (ii) eclosed as wasps, or (iii) died (±1 SEM). Sample sizes are shown in Table S1. Different letters in (a) indicate groups of species that had significantly different fly survival after exposure to *L. boulardi* ($z > 3.78$, $P < 0.003$). Pairwise comparisons were not carried out in (b) because most fly species had zero survival against *L. heterotoma*.

Fig. 3: Mean oviposition maintenance indices for female flies housed with (a) *L. boulardi* and (b) *L. heterotoma* (±1 SEM). Sample sizes are shown in Table S2. Oviposition maintenance indices equal to 1.00 (dashed lines) would indicate no difference in oviposition rates between control and wasp-exposed flies.

Fig. 4: Correlations between phylogenetically independent contrasts for: (a) fly oviposition maintenance indices vs. cellular immunity indices using *L. boulardi*, (b) fly oviposition maintenance indices vs. cellular immunity indices using *L. heterotoma*, (c) fly cellular immunity indices comparing *L. heterotoma* vs. *L. boulardi* infection, and (d) fly oviposition maintenance indices comparing *L. heterotoma* vs. *L. boulardi* exposure. *P* values from Pearson correlation tests are shown in each panel.

Fig. 5: (a) Mean cumulative per-female oviposition over 6 days for *D. yakuba* in food vials without wasps (control), with *L. boulardi* (Lb17-exposed), and with *L. heterotoma* (Lh14-exposed) (±1 SEM). (b) Mean proportions of *D. yakuba* larvae produced in each parental treatment that eclosed as flies: (i) with melanized capsules or (ii) without melanized capsules, along with total fly survival (i)+(ii), after being exposed to *L. boulardi* (±1 SEM). Sample sizes indicate number of vial replicates in (a) and number of dish replicates in (b).

Fig. 6: Mean cumulative per-female oviposition over 5 days in food vials without wasps (control), with *L. boulardi* (Lb17), and with *L. heterotoma* (Lh14) for different *D. melanogaster* strains: (a) *Oregon R* (wild-type), (b) w^{1118} (white-eyed), (c) GMR-*hid* (sight-deficient mutant), and (d) $Orco^{2}$ (olfaction-deficient mutant) (±1 SEM). The y-axis label is the same for all panels. N = 5 vial replicates per treatment per strain. Different letters indicate significant differences between treatments ($z > 3.44$, $P < 0.002$).

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SUPPORTING INFORMATION

Table S1: Cellular immunity dish replicates, total eclosion outcomes, and cellular immunity indices for each combination of fly and wasp species.

Table S2: Forced co-habitation vial replicates, cumulative per-female oviposition rates, and oviposition maintenance indices for each combination of fly and wasp species.

Table S3: Accession number and length of each amylase-related protein gene (*Amyrel*) coding sequence downloaded from GenBank.

Fig. S1: Ability of *D. melanogaster* and *D. simulans* offspring produced by control and wasp-exposed parents to survive wasp attacks.

Figure 1

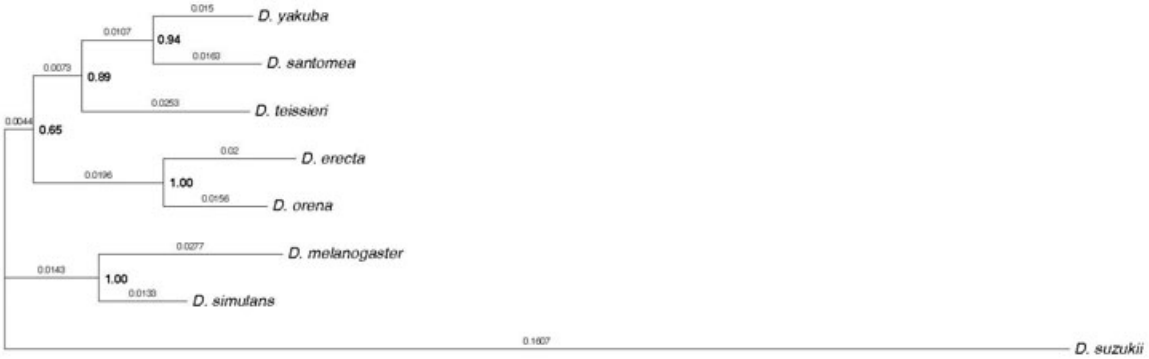

Figure 2

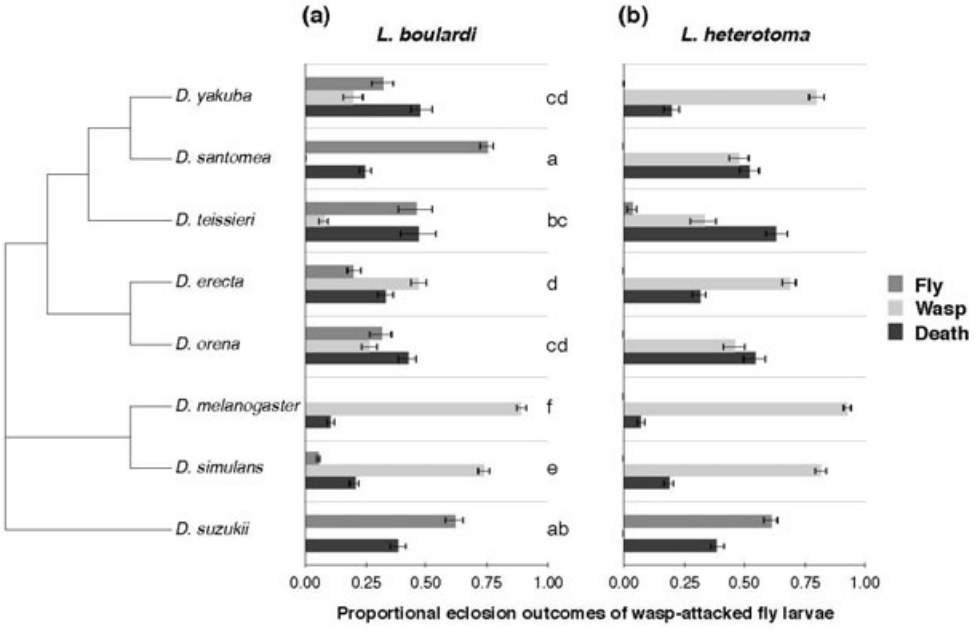

Figure 3

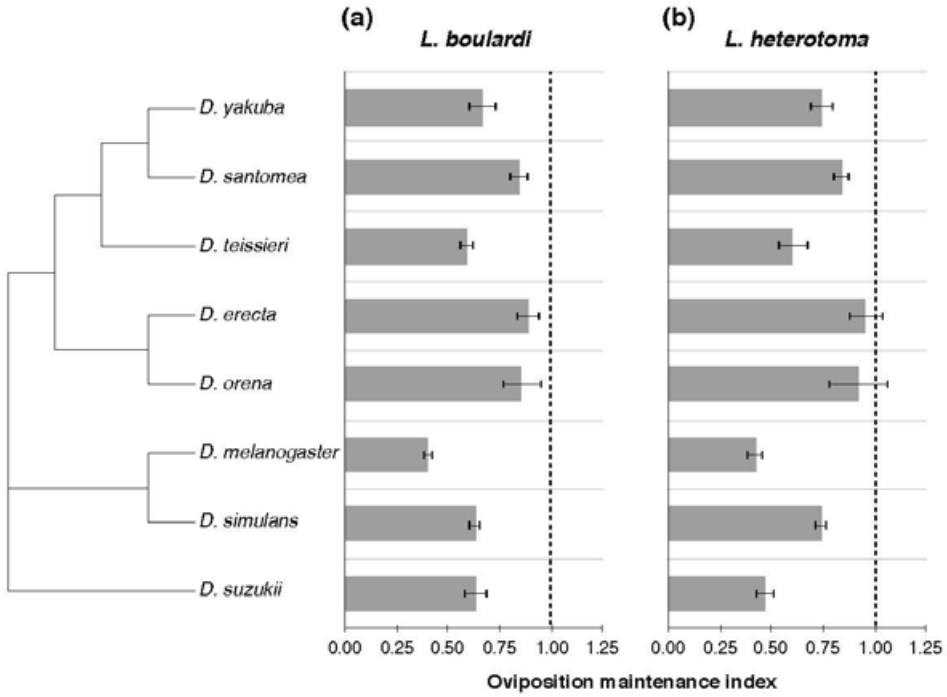

Figure 4

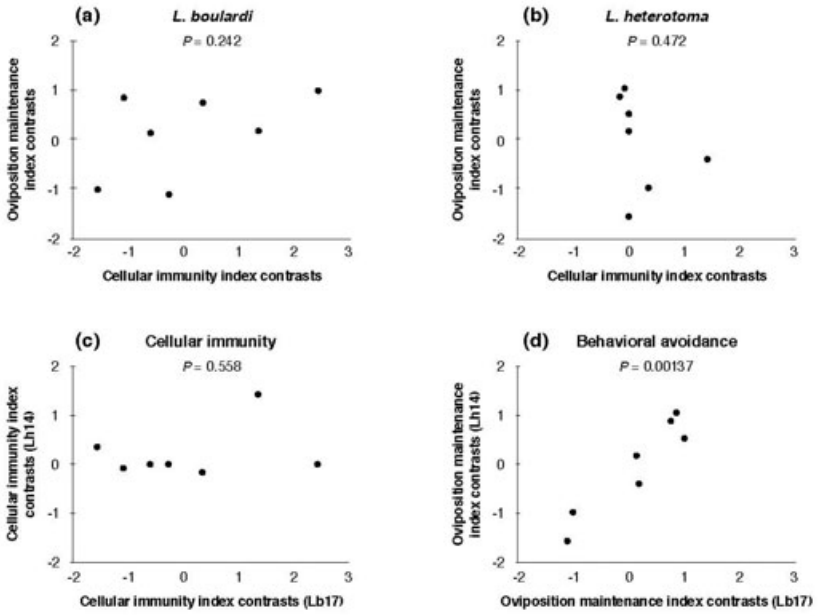

Figure 5

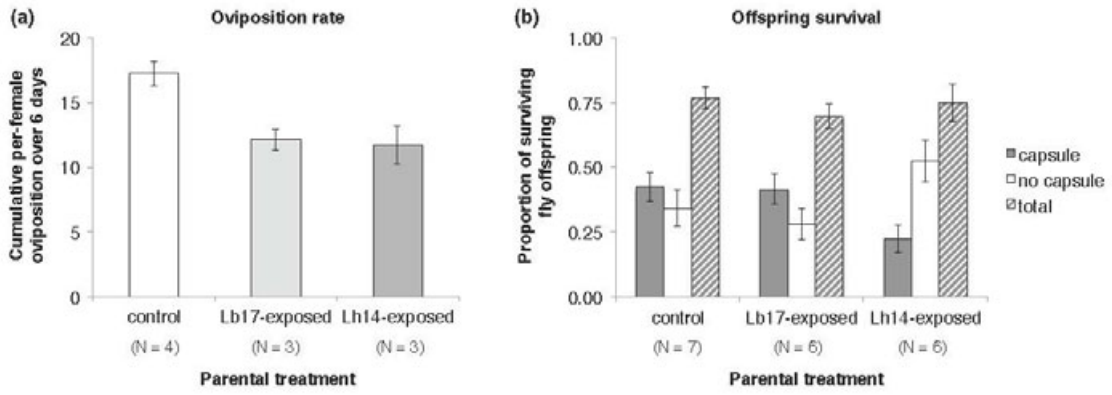

Figure 6