Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

______________________________ _______________

Ivan R. Shoemaker Date

Risk-Based Plasticity of Self-Medication Behavior in *Drosophila melanogaster*

By

Ivan R. Shoemaker

Master of Science

Graduate Division of Biological and Biomedical Science

Population Biology, Ecology & Evolution

____

Jacobus C. de Roode, Ph.D.

____

Christopher W. Beck, Ph.D.

____

Nicole M. Gerardo, Ph.D.

____

Todd A. Schlenke, Ph.D.

Accepted:

____

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

____

Date

Risk-Based Plasticity of Self-Medication Behavior in *Drosophila melanogaster*

By

Ivan R. Shoemaker

B.S., Salisbury University, 2006

M.S., Columbus State University, 2010

Advisor: Jacobus C. de Roode, Ph.D.

An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Graduate Division of Biological and Biomedical Science: Population Biology, Ecology & Evolution

2013

Abstract

Risk-Based Plasticity of Self-Medication Behavior in *Drosophila melanogaster*

By Ivan R. Shoemaker

Many insect hosts are capable of altering their behavior to reduce the probability, severity, or cost of infection by parasites. However, plasticity of behavior defense in response to varying risk of infection or fitness loss has rarely been addressed, and as a result, few cases have been reported. In the *Drosophila melanogaster* system, females provide trans-generational medication to their offspring when exposed to parasitoid wasps, and wasp-infected larvae self-medicate with ethanol. Yet, it is unclear from either study whether infected larvae or ovipositing adults seek specific ethanol concentrations or whether such a preference might vary in response to important risk factors, such as infection intensity, or as a result of interaction with different wasp species. By comparing the movement and survival of parasitized and uninfected *D. melanogaster* larvae in the presence and absence of ethanol, we find that *D. melanogaster* larvae do prefer particular ethanol concentrations that optimize their fitness, and that the magnitude of host self-medication response is plastic in response to infection intensity, host resistance, and ethanol effectiveness.

Risk-Based Plasticity of Self-Medication Behavior in *Drosophila melanogaster*

By

Ivan R. Shoemaker

B.S., Salisbury University, 2006

M.S., Columbus State University, 2010

Advisor: Jacobus C. de Roode, Ph.D.

A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Graduate Division of Biological and Biomedical Science: Population Biology, Ecology & Evolution

2013

TABLE OF CONTENTS

ABSTRACT………………………………………………...…………………………... iv

TABLE OF CONTENTS ………………………………………………..……………… v

LIST OF TABLES ………………………………………...…………………… vii

LIST OF FIGURES …………………….………………………..……………… viii

INTRODUCTION ………………………………………..…………………….. 1

MATERIALS AND METHODS…………………………………………...………. 5

Larval rearing and wasp exposure ...………………………….……...………. 5

Quantitative self-medication response of larvae to *L. heterotoma* infection ……..6

Adaptive value of self-medication response to *L. heterotoma* infection…………7

Infection intensity and self-medication response to *L. heterotoma* infection……..9

Specificity of ethanol-based self-medication behavior against parasitoid wasps…9

Statistical analysis……………………………………………………………..11

RESULTS AND DISCUSSION…………………………………………………………13

Quantitative self-medication response of larvae to *L. heterotoma* infection…….13

Adaptive value of self-medication response to *L. heterotoma* infection………...14

Infection intensity and self-medication response to *L. heterotoma* infection……15

Specificity of ethanol-based self-medication behavior against parasitoid wasps..16

CONCLUSION……………………………………………………………………..17

BIBLIOGRAPHY……………………………………………………………………18

LIST OF TABLES

Table		Page
1	Sixteen wasp strains used in this study represent 12 species from two families of hymenopteran parasitoids that commonly parasitize *Drosophila* larvae. Asterisks indicate wasps that required the use of prolonged exposure times and/or a greater number of ovipositing female wasps to obtain an 80% infection frequency.	25
2	Movement ratios, mean resistance scores, and mean benefit scores for larvae exposed to each wasp, as plotted in Figure 7. N=3 for resistance and benefit scores, except for Lh14 treatments, where N=5.	26

LIST OF FIGURES

Figure		Page
1	Schematic of foraging arenas. For each trial, ~120 exposed or unexposed larvae were introduced to a polystyrene arena containing food with a gradient of ethanol concentrations (A) or with no ethanol (B). Larvae were introduced to the center compartment and allowed to move and forage freely for 24 hours. When larvae were counted, numbers of larvae from mirroring compartments (e.g. 3% compartments) in each individual arena were summed and converted to proportions (i.e. p_0, p_3, p_6, and p_9), so that p_i represents the proportion of larvae found in compartment(s) containing i percent ethanol food. For no ethanol treatments, proportions of larvae were summed identically, even though compartments contained no ethanol. To calculate movement scores, proportions were multiplied by integers that indicated their distance from the center 0% ethanol compartment (i.e. 0, 1, 2, 3), summed, and divided by 3, the maximum possible value. Proportions and movement scores were calculated identically for ethanol gradient and no ethanol treatments.	27

2	Distribution of Lh14-exposed (A,B) and unexposed (C,D) *D. melanogaster* larvae on ethanol gradient (A,C) and no ethanol (B,D) food choice arenas. All pairwise comparisons between movement scores are significantly different, except between no ethanol treatments (panels B and D). N=10 for Lh14-exposed treatments, and N=5 for unexposed treatments. Error bars represent 95% confidence intervals.	28
3	Benefit and cost of ethanol medication demonstrated by successful eclosion of Lh14-exposed (A) and unexposed (B) *D. melanogaster* larvae reared on food containing 0, 3, 6, or 9% ethanol (i.e., "forced eclosions"). For each replicate, 30 larvae were introduced to 50 mL vials containing food with a single concentration of ethanol. N=5 for each treatment, and error bars represent 95% confidence intervals. The percent of larvae choosing each ethanol concentration (see Fig. 2a) was not significantly associated with successful eclosion of flies from Lh-14 exposed larvae (C), but the percent of unexposed larvae choosing each ethanol concentration (see Fig. 2c) showed a clear relation to eclosion success (D; $F_{1,2}$=203.3, p<0.01).	29
4	Benefit and cost of ethanol availability to Lh14-exposed and	30

unexposed *D. melanogaster* larvae, respectively, illustrated as successful eclosion of flies after being allowed to forage freely for 72 hours on ethanol gradient or no ethanol food choice arenas ("choice eclosions"). One-hundred larvae were used in each replicate, and food and larvae from all seven arena compartments were transferred to six ounce bottles for eclosion. Lh14-exposed larvae (A) experienced a significant increase in eclosion success when ethanol was available; however, no significant difference was observed among control larvae (B). N=5 for each treatment, and error bars represent 95% confidence intervals.

5 Infection intensity as a function of distance moved (from initial placement in the center 0% ethanol compartment; see Fig. 1) of Lh14-exposed D. melanogaster larvae after 24 hours. In food choice arenas with an ethanol gradient (A), compartment position was a significant predictor of mean number of wasp eggs dissected from individual larvae. No significant relationship was found for larvae introduced to arenas with no ethanol (B). The overall mean egg number per larvae was not significantly different between treatments. Five replicates were performed for each treatment. Error bars represent 1 SE, and sample sizes indicate the total number of larvae collected and dissected from each ethanol 31

concentration across all 5 replicates.

6 Distribution of *D. melanogaster* larvae on ethanol gradient and control food choice arenas when unexposed or exposed to various wasp species and strains. Mean movement scores are indicated for each wasp. N=3 for each treatment, except for unexposed and Lh14 treatments, where N=5. Lh14 plots are indicated by an arrow. Error bars represent 95% confidence intervals. Asterisks following wasp codes (see Table 1) indicate a significant difference between movement on arenas with an ethanol gradient and with no ethanol. Asterisks following movement scores within each plot indicate significant differences between the movement scores of exposed and unexposed larvae of the appropriate ethanol treatment. * p<0.05, **p<0.01, ***p<0.001 32

7 Movement ratios as a function of resistance and benefit for larvae exposed to 16 different wasps. Larval resistance (A), measured as the proportion of larvae eclosing with no ethanol, was significantly associated with movement; N=3 for each wasp, and error bars 95% confidence intervals. Ethanol effectiveness (B), measured as the sum of natural log-weighted differences in proportion of larvae eclosing, was also significantly associated with movement; N=3 for 33

each wasp, and error bars represent 1 SE. Filled circles and triangles represent the Figitid *Leptopilina* and *Ganaspis* species, respectively. Open circles and triangles represent the Braconid *Asobara* and *Aphaereta* species. Datapoint values are provided in Table 2, and plots with labeled points are provided in Figure 8.

8 Movement ratios as a function of resistance and benefit for larvae exposed to 16 different wasps. Larval resistance (A), measured as the proportion of larvae eclosing with no ethanol, was significantly associated with movement; N=3 for each wasp, and error bars 95% confidence intervals. Ethanol effectiveness (B), measured as the sum of natural log-weighted differences in proportion of larvae eclosing, was also significantly associated with movement; N=3 for each wasp, and error bars represent 1 SE. 34

9 Correlation between resistance, measured as the proportion larvae eclosing with no ethanol, and benefit or ethanol effectiveness, measured as the sum of natural log-weighted differences in proportion of larvae eclosing when given ethanol. N=3 for each wasp, and error bars represent 1 SE. Filled circles and triangles represent the figitid *Leptopilina* and *Ganaspis* species, respectively. Open circles and triangles represent the braconid *Asobara* and 35

Aphaereta species. Datapoint values are provided in Table 2.

INTRODUCTION

All free-living organisms host parasites (Windsor 1998) and, as a result of selection, should evolve mechanisms to reduce or recover losses in fitness caused by infection (Restif & Koella 2004). In invertebrates, hosts reduce or eliminate infection through a variety of physiological responses, including cellular encapsulation, melanization, and the production of humoral defense factors (Ratcliffe et al 1985). There is also increasing evidence that behavioral defenses play an important role in host immunity (de Roode & Lefèvre 2012). Hosts can alter their behavior to reduce the probability, severity, or cost of infection, and it has even been proposed that social hymenopterans have dispensed with a portion of canonical immune pathways in favor of these alternative behavioral defenses (Evans et al 2006, Bonasio et al 2010).

A wide range of behavioral defenses have been observed in insects. House flies (*Musca domestica*), for example, and several orthopterans exhibit behavioral thermoregulation, by which hosts seek out warm locations to raise their body temperatures to levels unfavorable to their parasites (Boorstein & Ewald 1987, Watson et al 1993, Inglis et al 1997, Elliot et al 2002). Alternatively, the bumblebee *Bombus terrestris* and several cockroach species seek out cooler temperatures for the same reason (Müller & Schmid-Hempel 1993, Moore & Freehling 2002). In social insects, such as termites, ants, and honeybees, grooming of relatives can increase resistance to infection at the individual or colony level (de Roode & Lefèvre 2012). Grooming behavior has also been documented in several solitary species. The eastern forktail *Ischnura vertaicalis*, a species of damselfly, is able to rid itself of ectoparasitic mites, and the Japanese beetle *Popillia japonica* uses grooming behavior to remove infectious nematodes (Gaugler et al

1994, Leung et al 2001). Some insects also tolerate infection through fecundity compensation. In response to infection with mites and bacteria, respectively, male *Drosophila nigrospiracula* and female house crickets (*Acheta domesticus*) increase early reproductive investment. Aphids hosting certain symbionts also increase reproductive investment when exposed to alarm pheromone (Barribeau et al 2010).

Medication behavior, though, is potentially the most documented and ecologically interesting of known insect behavioral defenses. Hosts employing this defense may exhibit either prophylactic medication, which is typically considered a constitutive behavior by which hosts prevent infection, or therapeutic medication, which includes behaviors that reduce or clear infection (de Roode and Lefèvre 2012). Wood ants, for example, exhibit prophylaxis by incorporating plant resin into their nests to inhibit fungal and bacterial growth (Castella et al 2008), whereas honeybees have been shown to increase resin foraging rates as a therapeutic response to immune challenge by the fungal parasite *Ascophaera apis* (Simone-Finstrom & Spivak 2012). Several species of caterpillar also demonstrate therapeutic medication by selectively foraging on otherwise harmful plant foliage in order to achieve resistance or tolerance against parasitoid flies (Karban & English-Loeb 1997, Singer et al 2009). Alternatively, trans-generational medication behavior can be used to reduce fitness losses by conferring resistance or tolerance to genetic relatives. In one example of this, the monarch butterfly *Danaus plexippus* is able to reduce the cost of infection by the protozoan parasite *Ophryocystis elektroschirra* by selectively ovipositing on larval host plants that increase both the resistance and tolerance of offspring (Lefèvre et al 2010).

Several types of medication behavior have also been described in *Drosophila* in response to infection by endoparasitoid wasps. Larvae of *D. melanogaster* preferentially feed on yeast species that increase successful resistance to parasitoids through cellular encapsulation (Anagnostou et al 2010). Mycophagous *Drosophila* species exploit the fungal toxin amanitin to increase the fitness of their offspring (Jaenike 1985), and more recently, female *Drosophila* were reported to provide trans-generational medication by preferentially ovipositing in ethanol-laden food (Kacsoh et al 2013). Larvae are less likely to be infected by nematodes or parasitoid wasps in the presence of amanitin and ethanol, respectively. Milan et al (2012) also showed that wasp-infected larvae self-medicate with food containing ethanol. However, it remains unclear whether infected larvae or ovipositing adults seek specific ethanol concentrations or whether such a preference might vary in response to important risk factors, such as infection intensity, or as a result of interaction with different wasp species.

Self-medication studies typically describe qualitative behavioral responses to infection (i.e. exhibiting behavior or not), but few have addressed the possibility that the magnitude of hosts' immune responses could vary as a function of infection intensity. In the monarch host-parasite system, Lefèvre et al (2010) investigated whether parasite load was associated with trans-generational medication behavior. The authors found that the adjustment of oviposition preference was inducible, but also that the response was fixed. However, if immune behaviors confer costs to hosts, selection should not only favor inducible behaviors, but also the evolution of defense behaviors that are plastic in magnitude of response to probability of infection, potential losses in fitness, or risk of mortality. In *Daphnia magna*, for example, the degree of fecundity compensation varies

in response to risk of infection by the microsporidian *Glugoides intestinalis* (Chadwick & Little 2005). Furthermore, if a defense provides protection against multiple enemies, the magnitude of response should again vary in relation to the risk posed by specific enemies. In this way, hosts might evolve highly effective defense mechanisms without incurring the total cost when the maximum level of defense is unnecessary or excessive.

Comparable trends have been observed in several predator-prey systems. Dipteran *Chironomus riparius* larvae increase burrowing behavior as a quantitative response to predator density (Hölker & Stief 2005), and *Rana lessonae* tadpoles exhibit continuous variation in defense behavior against predation risk by dragonfly larvae (Van Buskirk & Arioli 2002). Although not behavioral, morphologic transformation in defense structures in the ciliate *Eupotes daidaleus* is not only inducible by the presence of threatening predators, but also varies in response to different predators and predation risk (Kusch 1995, Altwegg et al 2004). Similarly, when the cost or probability of parasite infection varies in response to ecological factors, such as parasite density and identity, infection intensity, and G x G interactions, plasticity in host denfense(s) might be expected. However, such plasticity has not yet been reported in an insect host-parasite system.

In this study, we aim to assess 1) whether infected *D. melanogaster* larvae exhibit quantitative preferences for ethanol concentration, 2) whether preferences are adaptive, 3) whether the magnitude of self-medication response varies depending on infection intensity, and 4) whether medication behavior varies with host resistance and ethanol effectiveness against specific parasitoids. By comparing the movement and survival of parasitized and un-parasitized *D. melanogaster* larvae in the presence and absence of ethanol, we find that *D. melanogaster* larvae do prefer particular ethanol concentrations

that optimize their fitness, and that in addition to infection intensity, host resistance and ethanol effectiveness are both related to the magnitude of host self-medication response.

METHODS

Larval rearing and wasp exposure

Drosophila melanogaster strain Oregon R was used for all experiments. To obtain larvae, adult Oregon R flies were allowed to oviposit overnight on molasses food plates supplemented with yeast paste, and second instar larvae were collected ~48 hours following removal of adult flies. Both ovipositing adults and developing larvae were maintained at 25°C.

Most experiments were conducted with the wasp species *Leptopilina heterotoma*, strain Lh14, which was established from a single female collected from Winters, California, USA in 2002 (Schenke et al 2007) and has been maintained in the laboratory on *D. melanogaster* strain Canton S. Fifteen additional wasp strains, representing 11 species from two families of parasitoid wasps (Braconidae and Figitidae), were also used for experiments investigating the specificity of the larval self-medication response (Table 1). These wasps were collected from various locations and have been maintained on either *D. ananassae, D. melanogasater* strain Canton S, or *D. virilis* (Table 1), depending on which species of fly they are best able to infect.

In the following experiments, infected treatments were achieved by exposing second instar larvae to conditions conducive to infection. For experiments involving *L. heterotoma* strain Lh14, 30-40 female wasps were introduced to larval food plates and allowed to oviposit in the fly larvae for two hours, after which larvae were used in

foraging trials or eclosion experiments. For each replicate, a subset of larvae were dissected to ensure a minimum infection frequency of 80%, meaning that eight of 10 fly larvae contained at least a single wasp egg. Because it was impossible to confirm infection for larvae used in foraging trials or eclosion experiments, we hereafter refer to larvae as “exposed”, instead of “infected”. Exposure of larvae to the 15 additional wasps was conducted similar to Lh14 exposure, except that a number of species and/or strains with lower oviposition rates required prolonged exposure times (3-4 hours) and a greater number of female wasps (50-60) to obtain the desired infection frequency of 80% (Table 1).

Quantitative self-medication response of larvae to *L. heterotoma* infection

To test whether *D. melanogaster* larvae seek optimal concentrations of ethanol for medication against their parasitoid wasps, we constructed food choice arenas, in which larvae were allowed to move among and select from compartments filled with food that contained various concentrations of ethanol. Food choice arenas were constructed from 12.5cm x 8.5cm x 1.5cm polypropylene boxes, with 1cm x 8.5cm polypropylene dividers held in place by silicon sealant to create seven compartments (Fig. 1). Box lids had holes for airflow and were covered with fine cloth mesh to prevent escape of larvae. For no ethanol treatments, 1 gram of pulverized Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply Company) was added to each arena compartment and wet with 6 mL 1% red food dye solution (RDS). For ethanol gradient treatments, 1 gram of fly food was wet with 6 mL 0, 3, 6, or 9% v/v ethanol:RDS with 0% food at the center and concentrations increasing on either side in equal intervals (see Fig. 1). Ethanol concentrations were chosen to establish a gradient that included concentrations both less

and greater than 6% v/v, which had been used in previous experiments (e.g. Milan et al. 2012). In naturally maturing and decaying fruit, ethanol concentrations rarely exceed 4%, although concentrations of over 8% have been documented (McKechnie & Morgan 1982, Dominy 2004, Dudley 2004). Red food dye solution was used to facilitate counting larvae at the end of each trial by increasing the color contrast between experimental food and larvae.

At the start of each foraging trial, ~120 Lh14-exposed or unexposed larvae were placed in the central compartment of the arena. Larvae were kept at 25°C and allowed to move and forage freely within arenas for 24 hours. At the conclusion of each trial, the arena compartments were flooded with dH_2O, and larvae were counted in each. Ethanol gradient and no gradient Lh14-exposed treatments were replicated 10 times each, and 5 times each for unexposed treatments, for 30 trials in total, or ~3600 larvae.

In order to summarize the distribution of larvae within the arena compartments, movement scores were calculated for each replicate using the formula:

$$movement\ score = \frac{(0*p_0)+(1*p_3)+(2*p_6)+(3*p_9)}{3} \quad \text{Equation 1}$$

Each p_i value represents the proportion of larvae found in compartment(s) with i% ethanol food, or in corresponding compartments for no ethanol treatments (see Fig. 1). Proportions are weighted by multiplying each by integers that indicated the compartment that larvae were recovered from (i.e. distance from center compartment). The sum of the weighted proportions is then divided by 3, the maximum possible value (i.e., where p_9=1.0), in order to standardize the measure to a 0 to 1 scale.

Adaptive value of self-medication response to *L. heterotoma* infection

Although ethanol can be used as anti-wasp medication by fruit flies, it can also result in fitness costs due to its toxicity. To determine whether the observed quantitative response (i.e. movement score) was indeed adaptive, benefit and cost of ethanol were examined in relation to larvae choice in foraging trials. Survival to adulthood was measured as the percent eclosion, or emergence from pupal cases, of both exposed and unexposed *D. melanogaster* larvae, respectively, on different ethanol concentrations. For these "forced" eclosions, thirty Lh14-exposed or unexposed larvae were introduced into 50 mL cotton-plugged vials containing 0.75 grams of fly food wet with 4.5 mL 0, 3, 6, or 9% v/v ethanol:RDS. Successful eclosion for both flies and wasps was monitored twice weekly for four weeks, and mortality was calculated for each vial as the difference between the initial number of larvae introduced and the sum of emerging flies and wasps. Eclosion experiments on each ethanol concentration were replicated 5 times for both exposed and unexposed treatments.

To further demonstrate that conditional preference for ethanol provides an overall increase in larval fitness, the benefit and cost of ethanol availability was assessed by measuring the difference in eclosion success of Lh14-exposed or unexposed larvae, respectively, when allowed to forage freely on ethanol gradient or no ethanol food choice arenas prior to eclosion. For these "choice" eclosions, 100 Lh14-exposed or unexposed larvae were placed in the central compartment of a choice arena containing food with 0, 3, 6, and 9% v/v ethanol or containing no ethanol (see Fig. 1). The larvae and arenas were then incubated at 25°C for 72 hours, after which the food and larvae in all compartments from individual arenas were pooled and transferred to 6 ounce cotton-plugged bottles. Pooling compartments from ethanol gradient arenas was justified by the assumption that

the majority of ethanol had evaporated by 72 hours. This assumption was supported by our results, which demonstrated no significant difference in eclosion success of unexposed flies from ethanol gradient and no gradient arenas (1-way ANOVA, $p=0.1348$). Percent eclosion and death were determined as above, and each treatment was replicated 5 times.

Infection intensity and self-medication response to *L. heterotoma* infection

In order to determine whether the effect of infection intensity (i.e., more wasp eggs) on preference for ethanol concentration, larvae were dissected from five each of the ethanol gradient and five no ethanol foraging trials (N=509 and 581 larvae, respectively) described above. For each replicate, fly larvae from each compartment were individually dissected in Drosophila Ringer's solution (Ransom 1982) at 40x magnification. Wasp eggs are easily recognizable as distinct structures within the fly larvae under appropriate lighting and magnification. Again, data for larvae in compartments with equal ethanol concentrations, or equivalent positions in no ethanol trials, were pooled for each replicate.

Specificity of ethanol-based self-medication behavior against parasitoid wasps

Next, we assessed whether larval resistance or relative benefit of ethanol against specific wasps influences the degree of self-medication (i.e. movement scores) by *D. melanogaster* larvae. To determine resistance of *D. melanogaster* larvae to each wasp, additional "forced" eclosions were conducted for larvae exposed to each wasp (Table 1), with three replicates each. In these forced treatments, in which no ethanol was provided, eclosion despite infection indicates that larvae are able to kill wasp eggs or larvae via canonical physiological defense mechanisms, such as encapsulation or non-cellular

responses. As such, physiological resistance of fly larvae to each wasp was calculated as the mean proportion of wasp-exposed larvae eclosing in the absence of ethanol.

Alternatively, the relative benefit of ethanol medication can be defined as the increase in successful eclosion in the presence of increasing concentrations of ethanol. For each wasp, the changes in proportion of flies eclosing on 3, 6, or 9% v/v ethanol, relative to eclosion in the absence of ethanol, were weighted by ethanol concentration and summed, using the following equation:

$$benefit\ score = \log(3) * d_3 + \log(6) * d_6 + \log(9) * d_9 \qquad \text{Equation 2}$$

where $d_3 = p_3 - p_0$ and p_i represents the proportion of larvae successfully eclosing on food containing ethanol concentration i. Weighting d_i values by ethanol concentrations allows the benefit score to reflect the benefit provided by increasing levels of ethanol. Natural log-transformed ethanol concentrations were used in accordance to procedures used for calculating LD_{50} values.

To measure quantitative response to other wasps, food choice experiments were conducted with groups of larvae exposed to each of fifteen additional wasps. As described above for experiments with Lh14, ~120 wasp-exposed larvae were placed in the central compartment of ethanol gradient and no ethanol arenas, incubated for 24 hours, and larval placement used to calculate movement scores for each replicate. Both ethanol gradient and no ethanol treatments were replicated 3 times for each additional wasp, for a total of ninety additional foraging trials.

The degree to which fly larvae use ethanol medication against specific wasps was calculated as the relative change in mean movement score when on an ethanol gradient arena versus mean movement score on a no ethanol arena:

$$movement\ ratio = \frac{(m_{exp.eth}/m_{un.eth})}{(m_{exp.no}/m_{un.no})} \qquad \text{Equation 3a}$$

$$movement\ ratio = \frac{(m_{exp.eth}/0.14)}{(m_{exp.no}/0.32)} \qquad \text{Equation 3b}$$

$$movement\ ratio = 2.295\ \frac{m_{exp.eth}}{m_{exp.no}} \qquad \text{Equation 3c}$$

In Equation 3a, $m_{exp.eth}$ and $m_{exp.no}$ represent the mean movement scores of wasp-exposed larvae on ethanol gradient and no ethanol arenas, respectively. In order to incorporate changes in wasp-induced larval movement, even in the absence of an ethanol gradient, the mean movement scores of exposed larvae were corrected by dividing each by mean movement scores of unexposed larvae of the same ethanol gradient or no gradient treatment (Equation 3b), where $m_{un.eth}$ and $m_{un.no}$ represent the mean movement scores of unexposed larvae on ethanol gradient and no gradient arenas, respectively. Furthermore, because $m_{un.eth}$ and $m_{un.no}$ remain constant across all wasp species and strains, the equation can be simplified to Equation 3c.

Statistical analysis

To test whether fly larvae exhibit inducible ethanol-seeking behavior when infected by parasitoid wasps, movement scores for Lh14-exposed and unexposed larvae were compared using 1-way ANOVA and Tukey's HSD, and a 2-way ANOVA was used to test for interaction between wasp and ethanol treatment. One-way ANOVA and Tukey's HSD was also used to assess whether ethanol-seeking behavior was qualitative or quantitative by comparing percent larvae in each ethanol concentration of the exposed, ethanol gradient treatment (Fig. 2a).

In order to assess the relative benefit and cost of ethanol exposure in forced and choice eclosion experiments, differences in eclosion success between treatments were

assessed using a general linear model with a quasibinomial error distribution. Best models were identified using F-test comparisons. Relationships with larval preference were assessed by fitting to untransformed and log-transformed models, respectively.

To analyze the influence of infection intensity (wasp egg number) on ethanol concentration preference, the mean egg numbers dissected from fly larvae of compartments in each trial was examined as a function of the number of compartments moved from the starting point (i.e. 0, 1, 2, or 3). Considering a variety of models incorporating quadratic, square root, natural log, and natural log back-transformed terms, the best model was identified using AIC criteria. Optimum models were also confirmed using F-tests.

To determine whether *D. melanogaster* larvae respond similarly to infection by other parasitoid wasps, comparisons of movement scores between larvae exposed to additional wasps and unexposed larvae were also conducted using 1-way ANOVA. The influence of canonical physiological resistance and ethanol effectiveness on larval movement was assessed similarly to egg number. Models were optimized using AIC criteria and F-tests. Because single movement ratio values were calculated for larvae exposed to each wasp, both resistance and benefit score were examined as a function of movement ratio. Pearson's product-moment correlation coefficient was used to test for a relationship between resistance and benefit scores. AIC criteria and F-tests were also used to determine whether factors were redundant in their prediction of movement ratios.

Wherever appropriate, Shapiro-Wilk and Levene's tests were used to test for normality and homogeneity of variance, respectively. All statistics were performed using the R Statistical Package version 3.0.1 (R Core Team 2013).

RESULTS & DISCUSSION

Quantitative self-medication response of larvae to *L. heterotoma* infection

Our results confirm that Lh14-exposed *D. melanogaster* larvae exhibit a preference for ethanol-laden food. Significant differences were observed among the four treatment groups (1-way ANOVA, $F_{3,26}=44.14$, $p<0.001$), and Lh14-exposed larvae moved significantly more than unexposed larvae when introduced to an ethanol gradient (Fig. 2a,c; Tukey HSD, $p<0.001$). Unexposed larvae moved less on an ethanol gradient than without ethanol (Fig. 2c,d; Tukey HSD, $p<0.001$), suggesting a cost and innate avoidance of ethanol. Significantly more larvae preferred 0% to 3% ethanol compartments (Tukey HSD, $p<0.01$), and larvae preferred either more than 6% or 9% ethanol compartments (Tukey HSD, $p<0.01$). Lh14-exposed larvae, however, moved more on an ethanol gradient than in no ethanol arenas (Fig. 2a,b; Tukey HSD, $p<0.001$), suggesting that exposed larvae develop a conditional preference for ethanol. No significant difference was found between the movement of Lh14-exposed and unexposed larvae in no ethanol arenas (Fig. 2b,d; Tukey HSD, $p=0.3881$). A significant interaction was also observed between ethanol and wasp treatment (2-way ANOVA, $F_{1,24}=73.515$, $p<0.001$). These results indicate that Lh14-exposed flies do not simply move more than unexposed flies, but rather, exhibit an inducible preference for ethanol.

More larvae were counted in 3% and 6% compartments than at 0% ethanol, but no significant difference was found among proportions of infected *D. melanogaster* larvae at different ethanol concentrations. If larvae medicated in a qualitative manner, so that they only sought to increase their level of ethanol exposure, we would expect to

observe an equal or greater percent of larvae in 9% ethanol compartments; however, the percent larvae in 9% compartments was not significantly different from that in 0% or intermediate ethanol compartments (Fig. 2a; Tukey HSD). From these results, we are unable to conclude that larvae self-medicate in a quantitative manner, although the observation that a significantly higher percent of exposed larvae were found in 3% and 6% ethanol compartments than in 0% compartments might indicate a preference for intermediate ethanol concentrations (Fig. 2a; Tukey HSD, $p<0.05$).

Adaptive value of self-medication response to *L. heterotoma* infection

Consistent with previous findings (Milan et al 2012, Kacsoh et al 2013), we confirm that ethanol reduced the survival of unexposed larvae, as well as that the benefit of ethanol consumption outweighed fitness costs when larvae had been exposed to Lh14. In forced eclosion experiments, Lh14-exposed larvae had higher survival (greater percent of larvae successfully eclosed) on food containing ethanol than on food without ethanol, confirming a benefit of ethanol consumption for wasp-exposed flies. (Fig. 3a; $F_{2,9}=39.547$, $p<0.001$). In contrast, the unexposed larvae suffered a decrease in eclosion success with increasing ethanol levels (Fig. 3b; $F_{2,9}=100.16$, $p<0.001$). Thus, the relative cost or benefit of ethanol depends on infection status.

Plotting the eclosion success of larvae against their preferred ethanol concentrations in foraging trials (Fig. 2a,b) shows that fly larvae avoid ethanol concentrations that decrease their survival (Fig. 3d; $F_{1,2}=203.3$, $p<0.01$) and that, although not statistically significant, seem to prefer concentrations that optimize their fitness when previously exposed to Lh14 (Fig. 3c).

Choice eclosion experiments also demonstrated that larvae are able to conditionally exploit ethanol's medicinal properties when infected and are able to circumvent costs when uninfected. Lh14-exposed larvae experienced greater successful eclosion when presented with an ethanol gradient than without ethanol (Fig4a; $F_{1,8}$=26.23, p<0.001). Unexposed larvae, on the other hand, did not display a significant difference in eclosion when exposed to ethanol gradient or no ethanol treatments (Fig. 4b).

Infection intensity and self-medication response to *L heterotoma* infection

Our results show that greater infection intensity drives larvae to seek food with higher levels of ethanol. The distance that Lh14-exposed larvae moved from the center compartment of ethanol gradient arenas was a significant predictor of the number of wasp eggs they contained (Fig. 5a; $F_{1,18}$=504.1, p<0.001). In contrast, no significant relationship was found for Lh14-exposed larvae and distance moved on an arena without ethanol (Fig. 5b), thus indicating that infection intensity is unlikely to induce a general increase in movement by Lh14-exposed *D. melanogaster* larvae.

To our knowledge, this is the first reported instance of plasticity in an insect behavioral immune response. It remains unclear, though, whether higher ethanol concentrations are required to provide effective resistance against multiple wasp eggs/larvae. We hypothesize that this pattern is the result of an additive response to infection (i.e. egg number) and that such intensity-dependent response is maladaptive at higher levels of infection. The response has likely not been selected against in wild populations due to low frequency of super-parasitization or natural constraints on the maximum available concentration of ethanol.

Specificity of ethanol-based self-medication behavior against parasitoid wasps

Larvae that had been exposed to different wasps displayed considerable variation in mean movement scores (Fig. 6), and this was accompanied by significant variation in both resistance ($F_{15,32}$=24.102, p<0.001) and ethanol effectiveness ($F_{15,32}$=36.163, p<0.001). Only larvae exposed to *A. citri* ($F_{1,6}$=9.076, p<0.05), *A. japonica* ($F_{1,6}$=6.965, p<0.05), and *G. xanthopoda* strain GxUnk ($F_{1,6}$=7.375, p<0.05) elicited significant deviation from the movement of unexposed larvae, and none matched or exceeded the movement ratio of Lh14-exposed larvae. Interestingly, larvae exposed to some wasps displayed differential movement in the absence of ethanol (1-way ANOVA). *D. melanogaster* larvae exposed to *L. vicotoriae* strain LvHaw decreased movement in no ethanol treatments ($F_{1,6}$=6.587, p<0.05), while larvae exposed to *L. clavipes* strain LcNet displayed a significant increase in movement ($F_{1,6}$=9.163, p<0.05). In fact, in five of the eight wasps that elicited significantly divergent movement scores between larvae in ethanol gradient and no ethanol treatments, higher movement scores were observed for no ethanol treatments (Fig. 8).

Although few wasps elicited significant deviation from the movement of unexposed larvae (1-way ANOVA), our data clearly demonstrate that self-medication behavior was more apparent when larvae were less resistant to wasps. Movement ratio was significantly related to larval resistance to infection (Fig. 7a; $F_{1,46}$=46.907, p<0.001). When larvae were more resistant to a wasp, they medicated less, resulting in lower movement ratios. Movement was also associated with ethanol effectiveness (i.e. benefit score; Fig. 7b; $F_{1,46}$=26.447 p<0.001). When larvae were more likely to benefit from self-medication with ethanol, they were more likely to do so. The phylogenetic placement of

strains and species was not considered since there were no obvious trends regarding larvae response, and strains within species often produced more divergent responses than those observed between species, genera, and even families.

Finally, we found that resistance and ethanol effectiveness were highly correlated (Fig. 9; $r=0.8441$, $F_{1,14}=34.71$, $p<0.001$). Lower resistance was correlated with higher benefit, but the measures were not redundant, as models containing both predictors were significantly better than those containing either predictive variable alone.

CONCLUSION

Documenting alternative immune strategies and determining their underlying mechanisms is vital to understanding animal behavior, immune system evolution, and host-parasite interactions. In this study, we demonstrate risk-based plasticity in a self-medication defense behavior against parasitoid wasps. By comparing the movement and survival of parasitized and un-parasitized *D. melanogaster* larvae in the presence and absence of ethanol, we find that *D. melanogaster* larvae do prefer particular ethanol concentrations that optimize their fitness, and that in addition to infection intensity, host resistance and ethanol effectiveness are both related to the magnitude of host self-medication response. To our knowledge, this is the first reported instance of plasticity in insect behavioral immunity.

BIBLIOGRAPHY

Adamo, S.A. "The Specificity of Behavioral Fever in the Cricket *Acheta domesticus*." *Journal of Parasitology* 84, no. 3 (1998): 529-533.

Altwegg, R., K.B. Marchinko, S.L. Duquette, and B.R. Anholt. "Dynamics of an Inducible Defence in the Protist *Euplotes*." *Archiv fuer Hydrobiologie* 160, no. 4 (2004): 431-446.

Barribeau, S.M., D. Sok, and N.M. Gerardo. "Aphid Reproductive Investment in Response to Mortality Risks." *BMC Evolutionary Biology* 10 (2010): 251.

Bonasio, R., G. Zhang, C. Ye, N.S. Mutti, X. Fang, N. Qin, G. Donahue, P. Yang, Q. Li, C. Li, P. Zhang, Z. Huang, S.L. Berger, D. Reinbert, J. Wang, and J. Liebig. "Genomic Comparison of the Ants *Camponotus floridanus* and *Harpegnathos saltator*." *Science* 329 (2010): 1068-1071.

Boorstein, S.M., and P.W. Ewald. "Costs and Benefits of Behavioural Fever in *Melanoplus sanguinipes* Infected by *Nosema acridophagus*." *Physiological Zoology* 60 (1987): 586-595.

Van Buskirk, J., and M. Arioli. "Dosage Response of an Induced Defense: How Sensitive Are Tadpoles to Predation Risk." *Ecology* 83 (2002): 1580-1585.

Castella, G., M. Chapuisat, and P. Christe. "Prophylaxis with Resin in Wood Ants." *Animal Behavior* 75 (2008): 1591-1596.

Chadwick, W., and T.J. Little. "A Parasite-Mediated Life-History Shift in *Daphnia magna*." *Proceedings of the Royal Society B* 272 (2005): 505-509.

de Roode, J. C., and T. Lefèvre. “Behavioral Immunity in Insects.” *Insects* 3 (2012): 789-820.

Dominy, N.J. “Fruits, Fingers, and Fermentation: The Sensory Cues Available to Foraging Primates.” *Integrative and Comparative Biology* 44 (2004): 295-303.

Dudley, R. “Ethanol, Fruit Ripening, and the Historical Origins of Human Alcoholism in Primate Frugivory.” *Integrative and Comparative Biology* 44 (2004): 315-323.

Elliot, S.L., S. Blanford, and M.B. Thomas. “Host-Pathogen Interactions in a Varying Environment: Temperature, Behavioural Fever and Fitness.” *Proceedings of the Royal Society B* 269 (2002): 1599-1607.

Evans, J.D., K. Aronstein, Y.P. Chen, C. Hetru, J.-L. Imler, H. Jiang, M. Kanost, G.J. Thompson, Z. Zou, and D. Hultmark. “Immune Pathways and Defence Mechanisms in Honey Bees *Apis mellifera*.” *Insect Molecular Biology* 15 (2006): 645-656.

Gaugler, R., Y. Wang, and J.F. Campbell. “Aggressive and Evasive Behaviors in *Popillia japonica* (Coleoptera, Scarabaeidae) Larvae-Defenses Against Entomopathogenic Nematode Attack.” *Journal of Invertebrate Pathology* 64 (1994): 193-199.

Hölker, F., and P. Stief. “Adaptive Behaviour of Chironomid Larvae (*Chironomus riparius*) in Response to Chemical Stimuli from Predators and Resource Density.” *Behavioral Ecology and Sociobiology* 38, no. 3 (2005): 256-263.

Inglis, G.D., D.L. Johnson, and M.S. Goettel. “Effects of Temperature and Sunlight on Mycosis (*Beauveria bassiana*) (Hyphomycetes: Symopdulosporae) of Grasshoppers Under Field Conditions.” *Environmental Entomology* 26 (1997): 400-409.

Jaenike, J. “Parasite Pressure and the Evolution of Amanitin Tolerance in *Drosophila*.” *Evolution* 39, no. 6 (1985): 1295-1301.

Kacsoh, B.Z., Z.R. Lynch, N.T. Mortimer, and T.A. Schlenke. "Fruit Flies Medicate Offspring After Seeing Parasites." *Science* 339 (2013): 947-950.

Karban, R., and G. English-Loeb. "Tachinid Parasitoids Affect Host Plant Choice by Caterpillars to Increase Caterpillar Survival." *Ecology* 78 (1997): 603-611.

Kusch, J. "Adaptation of Inducible Defence in *Euplotes daidaleos* (Ciliophora) to Predation Risks by Various Predators." *Microbial Ecology* 30 (1995): 79-88.

Lefèvre, T., L. Oliver, M.D. Hunter, and J.C. de Roode. "Evidence for Trans-Generational Medication in Nature." *Ecology Letters* 13 (2010): 1485-1493.

Leung, B., M.R. Forbes, and R.L. Baker. "Nutritional Stress and Behavioural Immunity of Damselflies." *Animal Behavior* 61 (2001): 1093-1099.

McKechnie, S.W., and P. Morgan. "Alcohol Dehydrogenase Polymorphism of *Drosophila melanogaster*: Aspects of Alcohol and Tempmerature Variation in the Larval Environment." *Australian Journal of Biological Science* 35 (1982): 85-93.

Milan, N.F., B.Z. Kacsoh, and T.A. Schlenke. "Alcohol Consumption as Self-Medication Against Blood-Borne Parasites in the Fruit Fly." *Current Biology* 22 (2012): 488-493.

Moore, J., and M. Freehling. "Cockroach Hosts in Thermal Gradients Suppress Parasite Development." *Oecologia* 133 (2002): 261-266.

Müller, C.B., and P. Schmid-Hempel. "Exploitation of Cold Temperature as Defence Against Parasitoids in Bumblebees." *Nature* 363 (1993): 65-67.

Polak, M., and W.T. Starmer. "Parasite-Induced Risk of Mortality Elevates Reproductive Effort in Male *Drosophila*." *Proceedings of the Royal Society B* 265 (1998): 2197-2201.

R Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation

for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Ratcliffe, N.A., A.F. Rowley, S.W. Fitzgerald, and C.P. Rhodes. "Invertebrate Imunity: Basic Concepts and Recent Advances." *International Review of Cytology* 97 (1985): 183-329.

Restif, O., and J.C. Koella. "Shared Control of Epidemiological Traits in a Coevolutionary Model of Most-Parasite Interactions." *American Naturalist* 161 (2003): 827-836.

Simone-Finstrom, M.D., and M. Spivak. "Increased Resin Collection After Parasite Challenge: A Case of Self-Medication in Honey Bees?" *PLoS One* 7 (2012): e34601.

Singer, M.S., K.C. Mace, and E.A. Bernays. "Self-Medication as Adaptive Plasticity: Increased Ingestion of Plant Toxins by Parasitized Caterpillars." *PLoS One* 4 (2009): e4796.

Watson, D.W., B.A. Mullens, and J.J. Petersen. "Behavioral Fever Response of *Musca domestica* (Diptera: Muscidae) to Infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales)." *Journal of Invertebrate Pathology*. 61 (1993): 10-16.

Windsor, D.A. "Most of the species on Earth are parasites." *International Journal for Parasitology* 28 (1998): 1939-1941.

Table 1. Sixteen wasp strains used in this study represent 12 species from two families of hymenopteran parasitoids that commonly parasitize *Drosophila* larvae. Asterisks indicate wasps that required the use of prolonged exposure times and/or a greater number of ovipositing female wasps to obtain an 80% infection frequency.

Family	ID	Species	Locality	Date	Maintained On
Braconidae	Aph1Atl*	*Aphaereta sp.*	Atlanta, Georgia, USA	2009	*D. virilis*
	AcIC*	*Asobara citri*	Lambo, Ivory Coast	1995	*D. melanogaster*
	AjJap*	*Asobara japonica*	Tokyo, Japan	1995	*D. melanogaster*
	ApIndo*	*Asobara pleuralis*	Manado, Indonesia	2005	*D. melanogaster*
	AtFr*	*Asobara tabida*	Sospel, France	<1993	*D. virilis*
	AtSw*	*Asobara tabida*	Uppsala, Sweden	2007	*D. virilis*
Figitidae	GxUnk	*Ganaspis xanthopoda*	unknown	unknown	*D. melanogaster*
	G4Atl*	*Ganaspis sp.*	Atlanta, Georgia, USA	2011	*D. virilis*
	Lb17	*Leptopilina boulardi*	Winters, California, USA	2002	*D. melanogaster*
	LcAtl	*Leptopilina clavipes*	Atlanta, Georgia, USA	2011	*D. virilis*
	LcNet	*Leptopilina clavipes*	Heerenbergh, Netherlands	2000	*D. virilis*
	LgG500	*Leptopilina guineaensis*	Yaounde, Cameroon	1998	*D. melanogaster*
	LgG510*	*Leptopilina guineaensis*	False Bay, South Africa	1999	*D. virilis*
	Lh14	*Leptopilina heterotoma*	Winters, California, USA	2002	*D. melanogaster*
	LvHaw	*Leptopilina victoria*	Kaimuki, Hawaii, USA	2009	*D. ananassae*
	LvUnk	*Leptopilina victoria*	unknown	unknown	*D. ananassae*

Table 2. Movement ratios, mean resistance scores, and mean benefit scores for larvae exposed to each wasp, as plotted in Figure 7. N=3 for resistance and benefit scores, except for Lh14 treatments, where N=5.

	ID	Species	Movement ratio	Resistance (±95%CI)	Benefit (±95%CI)
Braconidae	Aph1Atl*	*Aphaereta sp.*	0.91	0.80 ± 0.06	-0.36 ± 0.26
	AcIC*	*Asobara citri*	3.97	0.21 ± 0.18	1.09 ± 0.44
	AjJap*	*Asobara japonica*	2.87	0.18 ± 0.10	0.05 ± 0.30
	ApIndo*	*Asobara pleuralis*	1.83	0.12 ± 0.07	0.39 ± 0.21
	AtFr*	*Asobara tabida*	1.06	0.32 ± 0.05	-0.07 ± 0.16
	AtSw*	*Asobara tabida*	0.74	0.71 ± 0.07	-1.04 ± 0.16
Figitidae	GxUnk	*Ganaspis xanthopoda*	1.35	0.16 ± 0.02	1.96 ± 0.05
	G4Atl*	*Ganaspis sp.*	4.04	0.44 ± 0.09	0.79 ± 0.10
	Lb17	*Leptopilina boulardi*	3.22	0.14 ± 0.05	0.50 ± 0.14
	LcAtl	*Leptopilina clavipes*	0.57	0.80 ± 0.23	-2.09 ± 0.56
	LcNet	*Leptopilina clavipes*	1.03	0.89 ± 0.10	-1.85 ± 0.43
	LgG500	*Leptopilina guineaensis*	2.74	0.11 ± 0.02	1.27 ± 0.04
	LgG510*	*Leptopilina guineaensis*	0.71	0.14 ± 0.05	0.83 ± 0.12
	Lh14	*Leptopilina heterotoma*	4.72	0.07 ± 0.00	1.17 ± 0.03
	LvHaw	*Leptopilina victoria*	4.64	0.04 ± 0.04	0.57 ± 0.12
	LvUnk	*Leptopilina victoria*	1.61	0.73 ± 0.04	-2.52 ± 0.34

Figure 1. Schematic of foraging arenas. For each trial, ~120 exposed or unexposed larvae were introduced to a polystyrene arena containing food with a gradient of ethanol concentrations (A) or with no ethanol (B). Larvae were introduced to the center compartment and allowed to move and forage freely for 24 hours. When larvae were counted, numbers of larvae from mirroring compartments (e.g. 3% compartments) in each individual arena were summed and converted to proportions (i.e. p_0, p_3, p_6, and p_9), so that p_i represents the proportion of larvae found in compartment(s) containing i percent ethanol food. For no ethanol treatments, proportions of larvae were summed identically, even though compartments contained no ethanol. To calculate movement scores, proportions were multiplied by integers that indicated their distance from the center 0% ethanol compartment (i.e. 0, 1, 2, 3), summed, and divided by 3, the maximum possible value. Proportions and movement scores were calculated identically for ethanol gradient and no ethanol treatments.

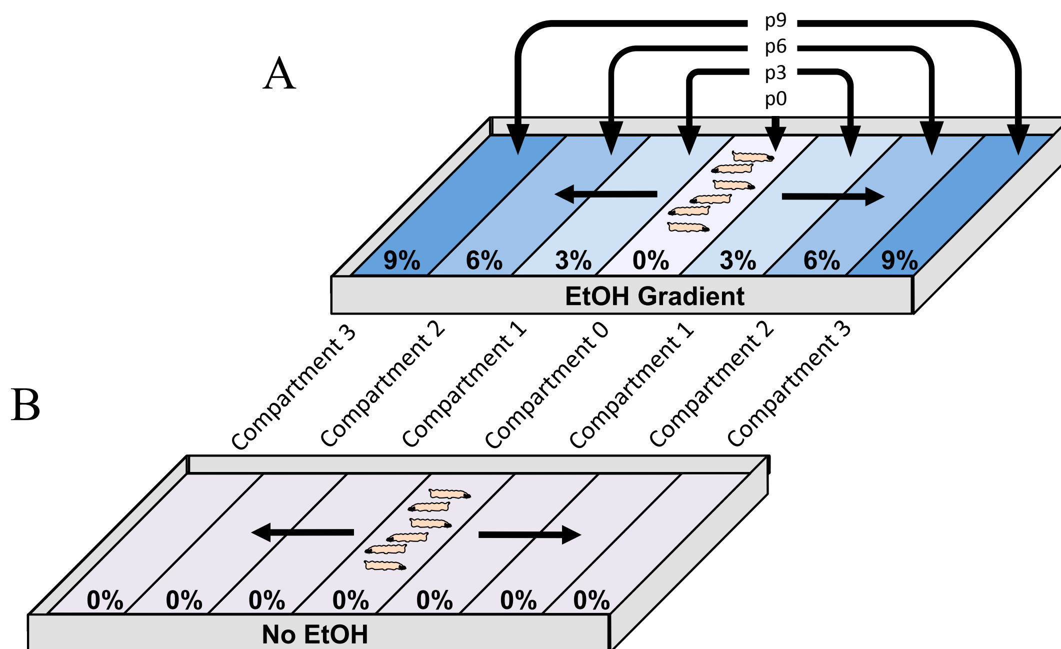

Figure 2. Distribution of Lh14-exposed (A,B) and unexposed (C,D) *D. melanogaster* larvae on ethanol gradient (A,C) and no ethanol (B,D) food choice arenas. All pairwise comparisons between movement scores are significantly different, except between no ethanol treatments (panels B and D). N=10 for Lh14-exposed treatments, and N=5 for unexposed treatments. Error bars represent 95% confidence intervals.

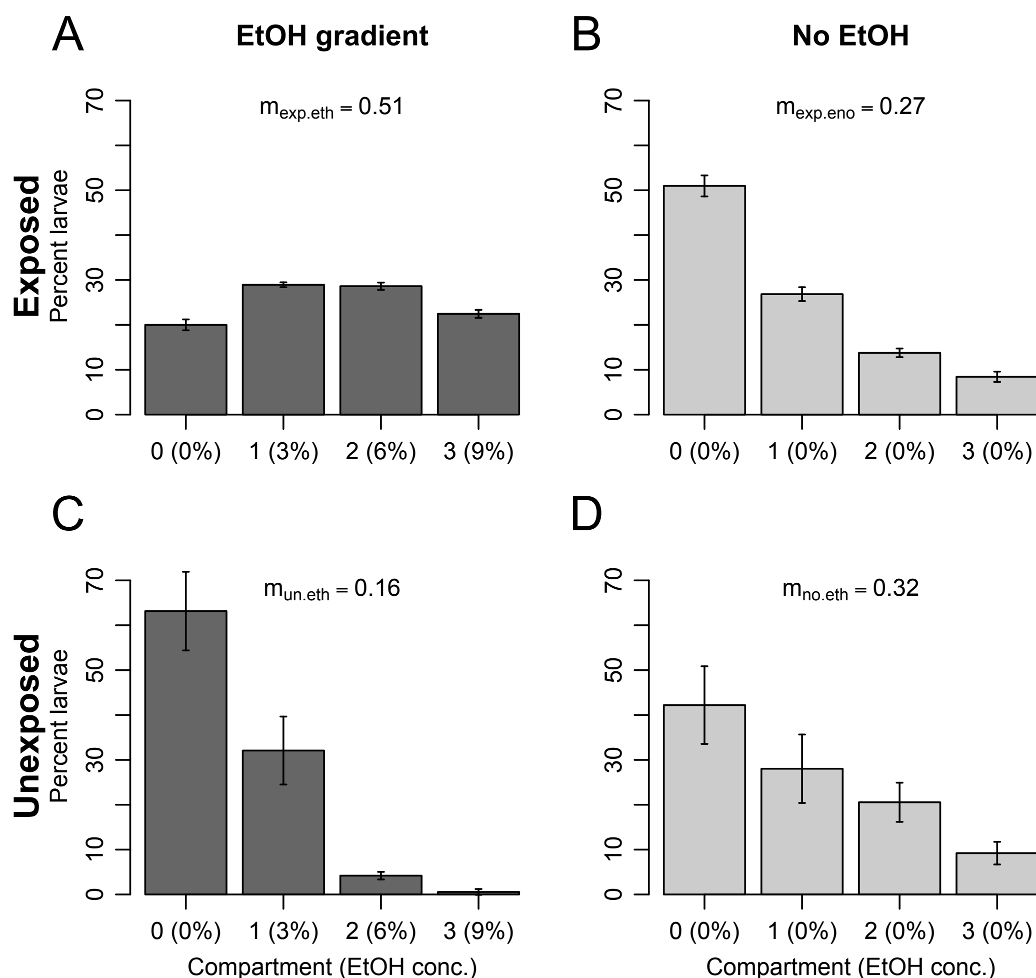

Figure 3. Benefit and cost of ethanol medication demonstrated by successful eclosion of Lh14-exposed (A) and unexposed (B) *D. melanogaster* larvae reared on food containing 0, 3, 6, or 9% ethanol (i.e., “forced eclosions”). For each replicate, 30 larvae were introduced to 50 mL vials containing food with a single concentration of ethanol. N=5 for each treatment, and error bars represent 95% confidence intervals. The percent of larvae choosing each ethanol concentration (see Fig. 2a) was not significantly associated with successful eclosion of flies from Lh-14 exposed larvae (C), but the percent of unexposed larvae choosing each ethanol concentration (see Fig. 2c) showed a clear relation to eclosion success (D; $F_{1,2}$=203.3, p<0.01).

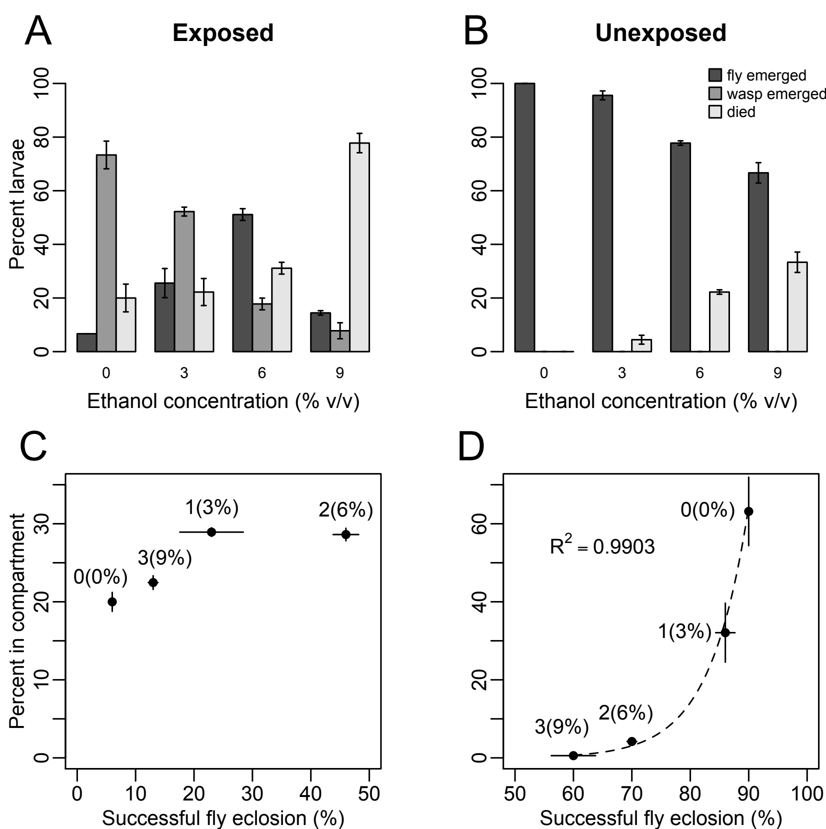

Figure 4. Benefit and cost of ethanol availability to Lh14-exposed and unexposed *D. melanogaster* larvae, respectively, illustrated as successful eclosion of flies after being allowed to forage freely for 72 hours on ethanol gradient or no ethanol food choice arenas ("choice eclosions"). One-hundred larvae were used in each replicate, and food and larvae from all seven arena compartments were transferred to six ounce bottles for eclosion. Lh14-exposed larvae (A) experienced a significant increase in eclosion success when ethanol was available; however, no significant difference was observed among control larvae (B). N=5 for each treatment, and error bars represent 95% confidence intervals.

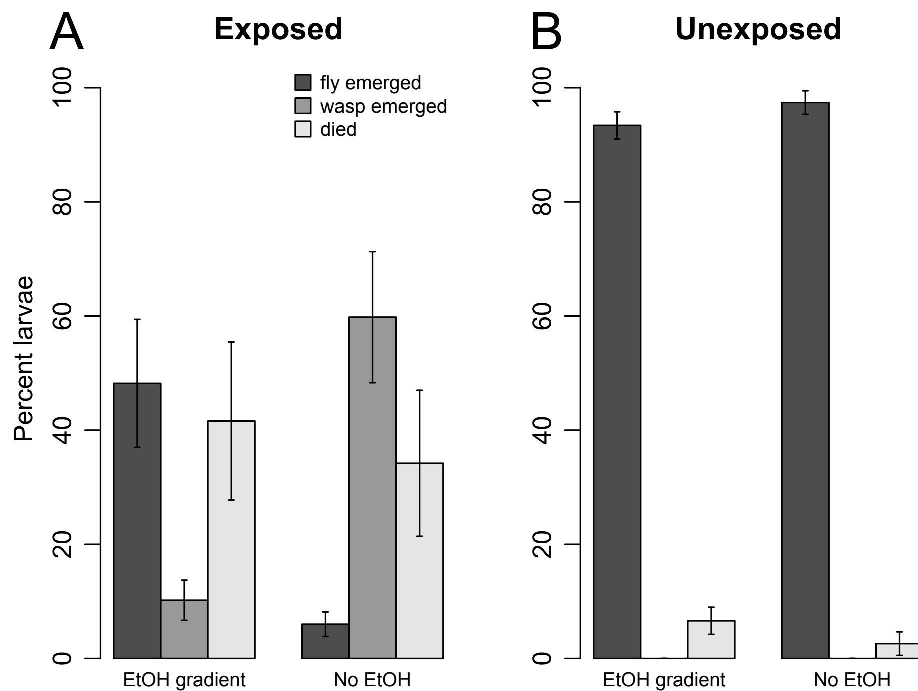

Figure 5. Infection intensity as a function of distance moved (from initial placement in the center 0% ethanol compartment; see Fig. 1) of Lh14-exposed D. melanogaster larvae after 24 hours. In food choice arenas with an ethanol gradient (A), compartment position was a significant predictor of mean number of wasp eggs dissected from individual larvae. No significant relationship was found for larvae introduced to arenas with no ethanol (B). The overall mean egg number per larvae was not significantly different between treatments. Five replicates were performed for each treatment. Error bars represent 1 SE, and sample sizes indicate the total number of larvae collected and dissected from each ethanol concentration across all 5 replicates.

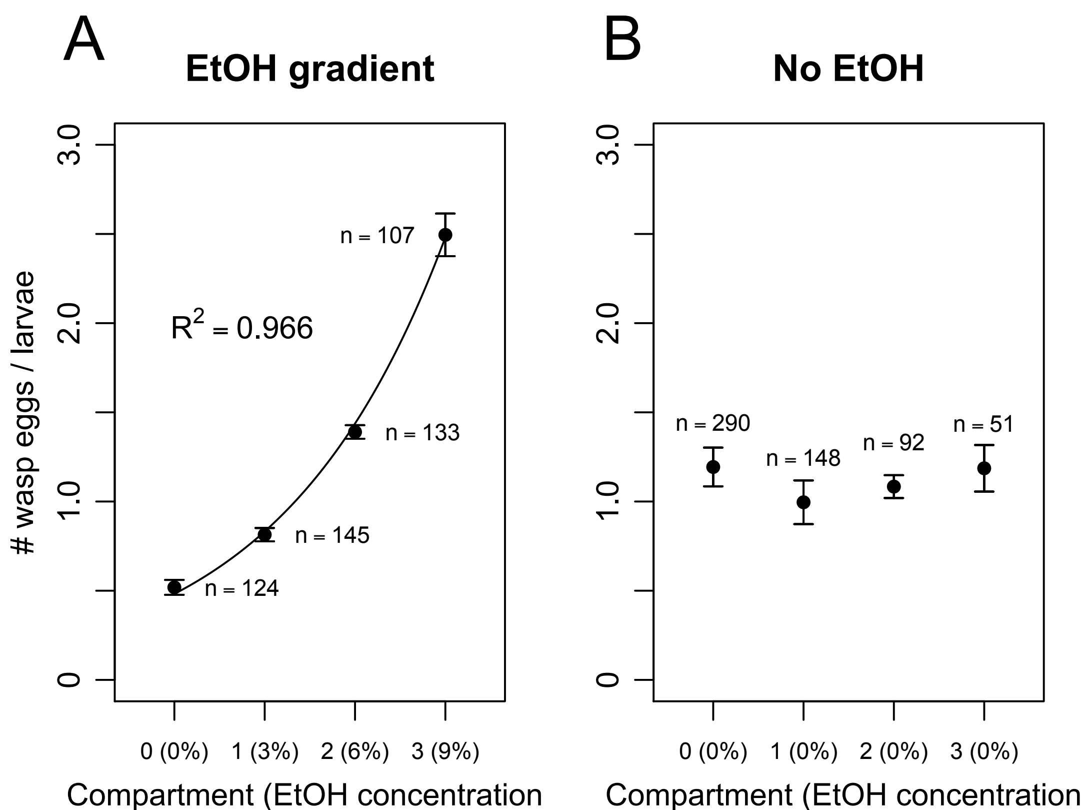

Figure 6. Distribution of *D. melanogaster* larvae on ethanol gradient and control food choice arenas when unexposed (see box) or exposed to various wasp species and strains. Mean movement scores are indicated for each wasp. N=3 for each treatment, except for unexposed and Lh14 treatments, where N=5. Lh14 plots are indicated by an arrow. Error bars represent 95% confidence intervals. Asterisks following wasp codes (see Table 1) indicate a significant difference between movement on arenas with an ethanol gradient and with no ethanol. Asterisks following movement scores within each plot indicate significant differences between the movement scores of exposed and unexposed larvae of the appropriate ethanol treatment. * p<0.05, **p<0.01, ***p<0.001

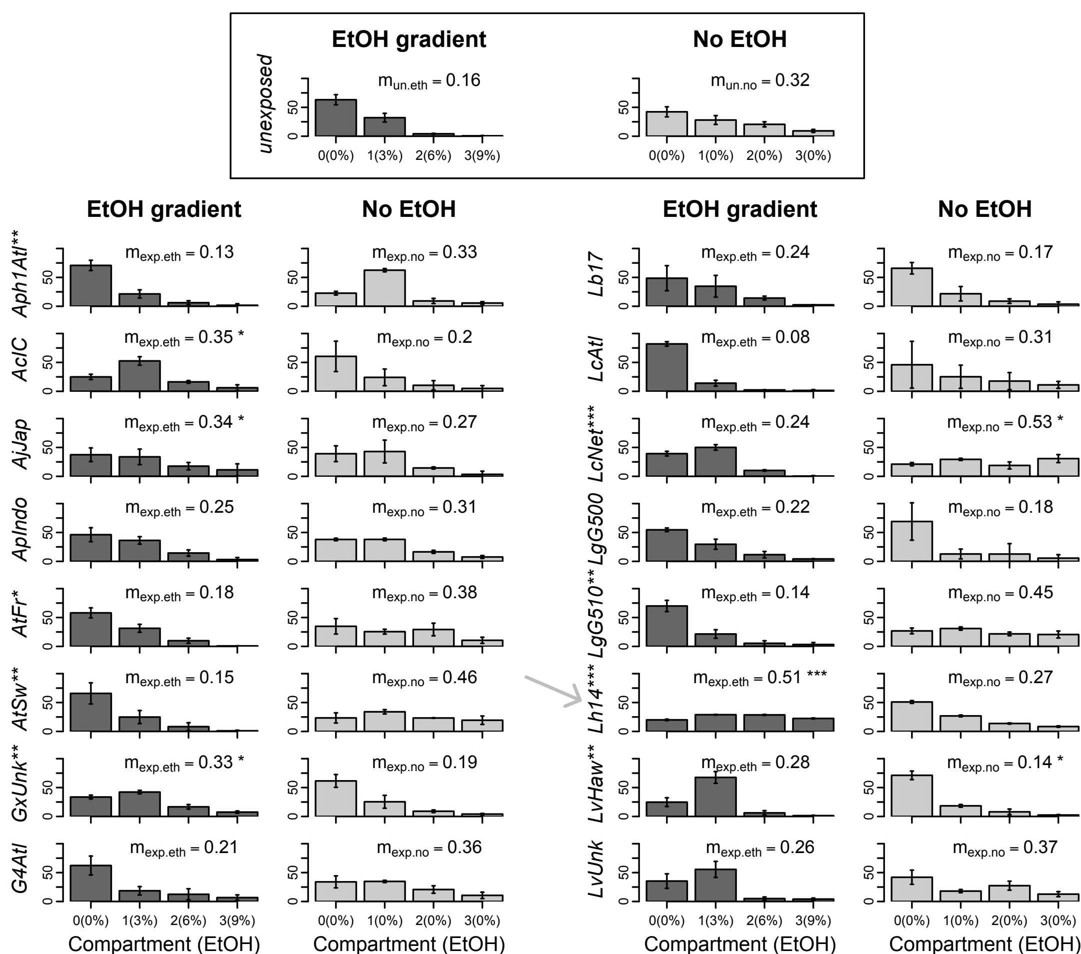

Figure 7. Movement ratios as a function of resistance and benefit for larvae exposed to 16 different wasps. Larval resistance (A), measured as the proportion of larvae eclosing with no ethanol, was significantly associated with movement; N=3 for each wasp, and error bars 95% confidence intervals. Ethanol effectiveness (B), measured as the sum of natural log-weighted differences in proportion of larvae eclosing, was also significantly associated with movement; N=3 for each wasp, and error bars represent 1 SE. Filled circles and triangles represent the figitid *Leptopilina* and *Ganaspis* species, respectively. Open circles and triangles represent the braconid *Asobara* and *Aphaereta* species. Datapoint values are provided in Table 2, and plots with labeled points are provided in Figure 8.

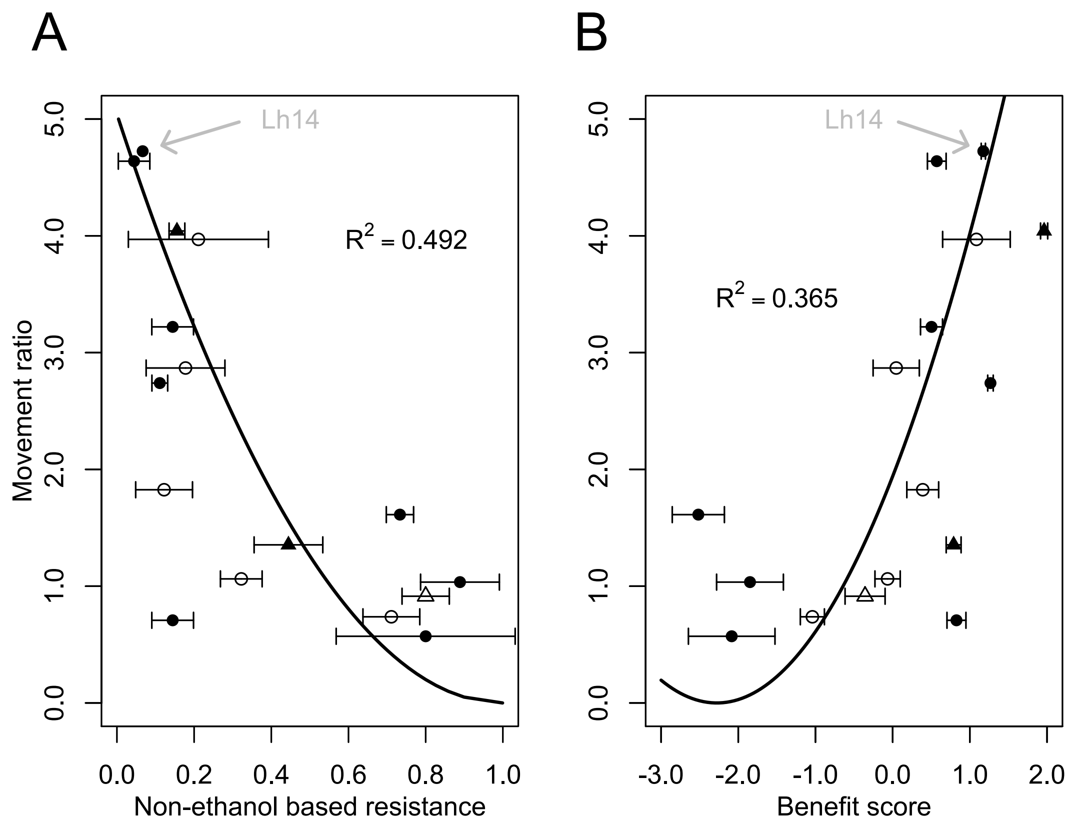

Figure 8. Movement ratios as a function of resistance and benefit for larvae exposed to 16 different wasps. Larval resistance (A), measured as the proportion of larvae eclosing with no ethanol, was significantly associated with movement; N=3 for each wasp, and error bars 95% confidence intervals. Ethanol effectiveness (B), measured as the sum of natural log-weighted differences in proportion of larvae eclosing, was also significantly associated with movement; N=3 for each wasp, and error bars represent 1 SE.

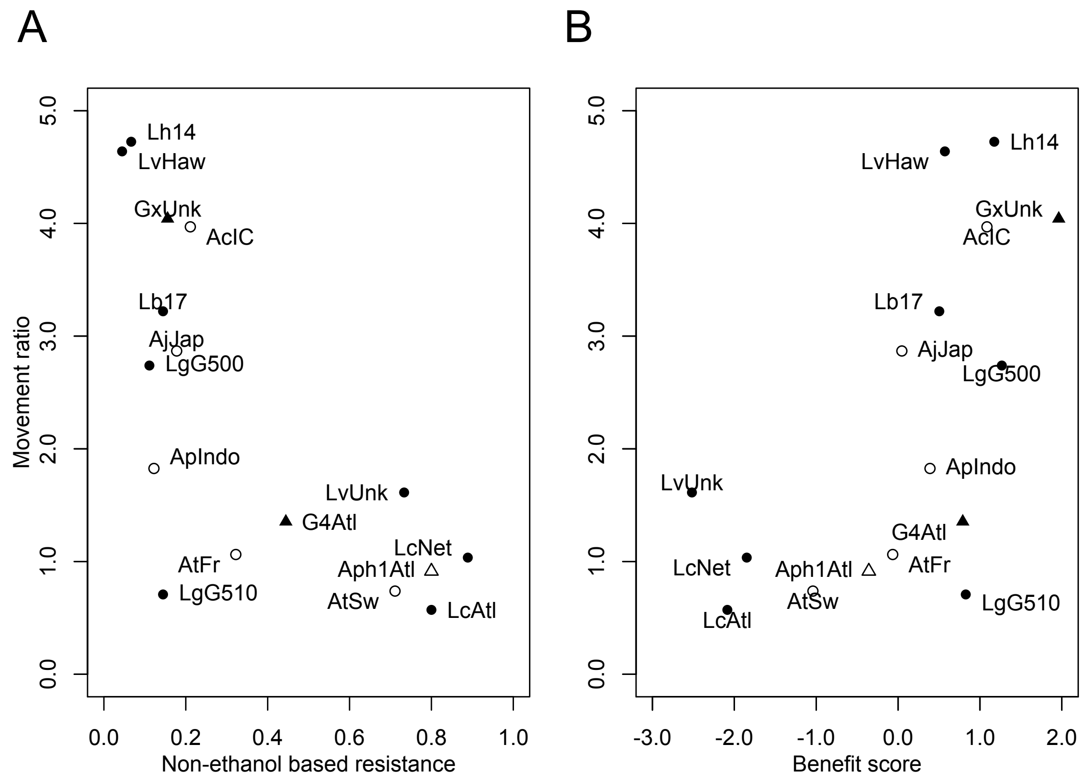

Figure 9. Correlation between resistance, measured as the proportion larvae eclosing with no ethanol, and benefit or ethanol effectiveness, measured as the sum of natural log-weighted differences in proportion of larvae eclosing when given ethanol. N=3 for each wasp, and error bars represent 1 SE. Filled circles and triangles represent the figitid *Leptopilina* and *Ganaspis* species, respectively. Open circles and triangles represent the braconid *Asobara* and *Aphaereta* species. Datapoint values are provided in Table 2.