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Changes in immune effort of male field crickets infested with mobile parasitoid larvae

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Abstract

Insect immune defenses include encapsulation and the production of lysozymes and phenoloxidase. However, the highly mobile larvae of parasitoid Ormiine flies (*Ormia ochracea*) can evade initial encapsulation, and instead co-opt host immune responses to form a critical respiratory funnel connecting them to outside oxygen. Here we ask how field crickets (*Teleogryllus oceanicus*) respond immunologically to *O. ochracea* infestation. Host encapsulation and phenoloxidase play important roles in the formation of the respiratory funnel, so we hypothesized that decreases in these immune parameters during infestation may interfere with respiratory funnel formation and increase the likelihood of larval death. Encapsulation ability decreased after infestation with *O. ochracea* larvae, but phenoloxidase activity increased in both infested crickets and controls, whereas lysozyme activity decreased in infested crickets but remained constant in controls. Hosts with fewer established larvae showed greater decreases in encapsulation, and phenoloxidase activity was positively associated with the degree of larval respiratory funnel melanization. Differences between phenoloxidase and lysozyme activity in infested crickets are consistent with a trade-off within the immune system of hosts, and our results demonstrate the effects of a prior immune challenge on the ability to mount a subsequent response.
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1. Introduction

Parasitoid infection in insects represents a unique type of challenge to host immune defenses. Failing behavioral evasion of parasitoids (Lewkiewicz and Zuk, 2004) or cuticular barriers (Vinson, 1990), hosts must swiftly cope with infestation using an array of immune defenses. Hosts are expected to maximize immunological defenses involved in early detection and elimination of parasitoids, because once established, parasitoids kill their hosts (Godfray, 1994). Most work examining such defenses has focused on hymenopteran parasitoids, which inject small, stationary eggs into the body cavities of their hosts (Meister and Lagueux, 2003; Gwynn et al., 2005; Lu et al., 2006). Fly parasitoids in the family Tachinidae, however, present a drastically different challenge because they infest their hosts with highly mobile larvae (Clausen, 1940).

The primary line of defense insect parasitoids must overcome is the ability of their host to encapsulate foreign invading objects with a crust of hemocytes that die and then melanize (Vinson, 1990; Gupta, 1991; Strand and Pech, 1995; Lavine and Strand, 2002). Encapsulated invaders are killed by a combination of asphyxiation and exposure to cytotoxic compounds such as quinones, semiquinones and free radicals, which are produced during melanin formation (Meister and Lagueux, 2003; Cerenius and Söderhäll, 2004). A key component of the encapsulation response is phenoloxidase (PO), which is a commonly measured intermediary in the formation of melanin (Söderhäll and Cerenius, 1998; Adamo, 2004a; Cerenius and Söderhäll, 2004).

Flies of the larviparous Tachinid species *Ormia ochracea* are common parasitoids of North American field crickets. Gravid females locate hosts by moving towards calling males, whereupon they spray first instar larvae, or planidia, onto them (Cade, 1975; Wineriter and Walker, 1990). After burrowing into the host's thorax, planidia migrate into the

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abdominal cavity and proceed through three instars. Cutaneous absorption of oxygen is insufficient to meet the needs of rapidly growing larvae, and second instar larvae attach themselves to the abdominal wall of their host and create a perforation to the outside using their posterior spiracles (Clausen, 1940). In *O. ochracea*, this occurs 3 days after infestation (Adamo et al., 1995). The host responds to this mechanical injury by initiating a pigmented, scab-like growth of epidermal origin which encloses the posterior end of the attached larva (Clausen, 1940; Beard, 1942; Salt, 1963). As the larva grows, this enveloping membrane grows longitudinally and radially with it, forming a respiratory funnel that connects the posterior one or two segments of the larva to the hole in the abdominal wall (Salt, 1968; Vinson, 1990; Godfray, 1994; Adamo et al., 1995). The respiratory funnel is a common feature of most tachinid larvae. An additional translucent casing extends from the pigmented basal portion of the respiratory funnel and envelopes much of the larval body in many species, including *O. ochracea*, and has been termed the 'respiratory sheath' (Salt, 1963). Most lines of evidence suggest that the sheath is derived from encapsulating hemocytes that surround the larva and die, forming a flat, thin membrane (Salt, 1963). Continued growth of the respiratory funnel forces the larva to detach from its initial connection to the abdominal wall, but the respiratory funnel and sheath serve as an anchor to the outside source of oxygen and an additional barrier to immunological compounds produced by the host (Clausen, 1940; Beard, 1942; Salt, 1963, 1968; Thorpe and Harrington, 1979).

The degree of melanization of the pigmented basal portion of the respiratory funnel is expected to be related to the host's PO activity, because PO is involved in the sclerotization and tanning of damaged cuticular tissue (Andersen, 1985). Ashida and Brey (1995) showed that cuticular PO originates in the host hemolymph, where it is stored as the inactive precursor, pro-PO. Levels of cuticular PO and hemolymph-bound PO are also highly correlated (Cotter and Wilson, 2002), and we expected that the extent of larval respiratory funnel melanization would depend on the amount of host pro-PO available in the hemolymph. Similarly, the efficacy of the respiratory sheath may depend on the encapsulation response of the host. Decreased encapsulation would be expected to weaken the respiratory sheath, increasing the likelihood that larvae might break free during feeding activity and asphyxiate. A weakened respiratory sheath would also increase larval exposure to cytotoxic compounds produced during melanin formation, as well as other antimicrobial compounds such as lysozymes. Second-instar larvae are dependent on both host encapsulation and PO responses to construct this critical respiratory connection, so populations heavily parasitized by Ormiine flies may—counter-intuitively—decrease these responses (Zuk et al., 2004).

Here we use artificial infestations to examine how field crickets respond immunologically to infestation by parasitoid fly larvae. The Australian and Pacific island-distributed field cricket *Teleogryllus oceanicus* is subject to parasitism by *O. ochracea* where their ranges overlap in Hawaii (Otte and Alexander, 1983; Zuk et al., 1993). Levels of parasitism are high in Hawaii, with up to 30% of males harboring parasitoid larvae, whereas only 7% of females are parasitized (Zuk et al., 1993). We therefore assessed encapsulation, PO activity and lysozyme activity (LA) in artificially infested *T. oceanicus* males. To control for individual variation in immunity, we chose an experimental design that allowed us to assess host immunity just prior to infestation and 3 days after infestation, and we used a control group of uninfested crickets to examine and separate the effects of a prior immune challenge. The timing of infestation was key, because we were interested in how crickets respond immunologically during the narrow timeframe when larvae enter the abdominal cavity and begin co-opting their host's defenses to construct a respiratory funnel. Are host encapsulation and PO responses increased or decreased at this point? Decreased host encapsulation and PO activity may maximize the likelihood of killing *O. ochracea* larvae late during infestation by interfering with respiratory funnel formation. We therefore predicted that: (1) stronger encapsulation and PO responses 3 days after infestation would be associated with greater numbers of larvae developing within hosts; (2) crickets with greater encapsulation and PO responses would harbor larvae with more heavily melanized respiratory funnels; and (3) LA would remain constant before and after infestation, since it is involved in defense against bacterial pathogens (Schneider, 1985), but not larger invading bodies.

2. Materials and methods

2.1. Cricket and fly collection

We used *T. oceanicus* males from a lab colony established from individuals collected in 1998 at the University of Hawaii at Manoa on the island of O'ahu, and supplemented yearly from the same source. Crickets were reared on a 12:12 light:dark cycle at 30 °C in 15 L plastic containers and were provided Purina rabbit chow and water *ad libitum*. To control for any effects of mating history on immunity (Shoemaker et al., 2006), we ensured the virginity of males used in this study by monitoring boxes of nymphs at least every other day and removing newly eclosed males to a new container each time. We collected males in this manner for 4 weeks prior to the start of the experiment, to produce a cohort of virgin males of known ages.

Collecting and transporting flies from Hawaii was prohibitively difficult. Mitochondrial sequence data indicates that haplotypes from *O. ochracea* populations in Hawaii and the southwestern United States are very similar (D. Gray, unpublished data). We therefore collected flies from the Santa Monica National Recreation Area in

Thousand Oaks, CA in September 2006 (Permit#: SAMO-2006-SCI-0019).

Synthesized calling song from *T. oceanicus* males was broadcast from funnel-style sound traps starting at approximately 7:00 PM. Captured flies were immediately transported to the lab where they were housed in plastic containers with twigs, cotton soaked in water, applesauce, sugar cubes and powdered milk.

2.2. *Artificial infestation and immune response assays*

We assessed the immune responses of males before infestation with *O. ochracea* larvae and 3 days after infestation, and a control group was assessed in the same manner, except they were not infested. Repeated measures ANOVAs were used to explore the effects of infestation on changes in each immune response. We tested all data for deviations from normality prior to using ANOVAs. None were found. The relationship between the number of larvae harbored and the immune parameters assayed was also examined.

At the start of the experiment, 4 μL of hemolymph was withdrawn from each of 152 male crickets through a small puncture between the second and third sternite on the left side of the lower abdomen. The hemolymph was deposited into 55 μL of 1 × PBS (11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, pH = 7.4, Fisher) and kept on ice briefly until being stored at -80 °C. Following the protocol of Fedorka et al. (2004), we inserted into the wound a 3 mm removable nylon monofilament implant with a knot at the external end that allowed for its later removal. Implants were abraded with sandpaper to increase the likelihood of hemocyte adhesion. We housed each implanted cricket individually in a 236 mL container with food, a water vial, and egg carton for shelter (changed daily throughout the experiment). After 36 h, the implant was removed and stored at −10°C.

We allowed the crickets to rest for 12 h after removing the implant before we infested them with *O. ochracea* larvae. By that time, their previous wound had sealed. First instar planidia were dissected from the gravid female flies, and three were transferred onto each cricket under the junction between the thorax and the abdomen. Crickets were infested with three planidia to simulate a realistic level of infestation. *T. oceanicus* males can harbor more than three larvae in the wild, but no more than two have been observed to emerge (Zuk et al., 1993). Crickets in the control group were not infested, but were handled the same manner. Seventy-seven crickets were infested, and 38 were uninfested controls. The two treatment groups contained crickets of equally mixed ages. The first implant either fell out of or was removed by 37 crickets, and these were therefore eliminated from the experiment.

The nylon implant and hemolymph withdrawal procedures were repeated on both infested and control crickets 72 h after infestation, except this time the right side of the lower abdomen was punctured to avoid inserting an implant into an already-scarred wound. At this time, 10 infested crickets were dissected to assess what stage of development the larvae had achieved. After 36 h, all nylon implants were removed as before. When the first larvae began to emerge from infested crickets (6 days after infestation), each cricket was measured (head capsule width) and dissected to ascertain the number of larvae it had harbored.

The average wet weight of a sample of males from this lab population was 587 mg (N. Bailey, unpublished data). Zera and Holtmeier (1992) estimated total hemolymph volume in comparably sized field crickets, *Gryllus rubens*, to be between 19% and 22% for crickets older than 6 days. Using the most conservative estimates, the amount of hemolymph withdrawn at any given time during the present experiment was a negligible proportion of the total hemolymph volume per cricket (less than 5%).

2.2.1. *Encapsulation assay*

Encapsulation of the removable nylon implants was assessed using a technique adapted from Rantala et al. (2003), Rantala and Kortet (2003), Fedorka et al. (2004) and Zuk et al. (2004). The knot at the end of each encapsulated nylon filament was cut off, and the implants were gently wiped to remove any extraneous fat body or tissue that may have been attached during removal. Melanization of the implants was quantified by photographing them with a RT Color Spot digital camera attached to a Leica MZ75 stereomicroscope under 3.2 × magnification. *T. oceanicus* crickets encapsulate implants unevenly, which results in the heaviest, most melanized side of the implant rolling downwards when placed on a microscope slide. To reduce measurement variation, we systematically placed each implant darkest-side up on the slide before photographing it. The darkness of each implant was assessed using macros in the program NIH Image (v.1.62) that first outline all areas above a certain gray-scale value, and then calculate a mean value for the darkness of the outlined area, with 0 being completely white and 256 being completely black. The aperture of the stereomicroscope was kept constant across all photographs, as was the intensity of light transmitted through the mounted tips from below the stage plate, thereby allowing a direct comparison of the darkness values calculated for each implant. Measuring the darkness of encapsulated implants is a commonly used technique for assaying immunity in insects, and has been found in previous studies to consistently reflect the number of hemocytes used during an immune challenge (Baer et al., 2006).

2.2.2. *Phenoloxidase assay*

PO is stored in hemolymph as an inactive precursor, pro-PO. During an immune challenge, pro-PO is cleaved by a proteolytic enzyme to form PO (Ashida and Yamazaki, 1990). In this study, we adapted protocols from Adamo (2004a), Fedorka and Zuk (2005) and Shoemaker et al.

(2006) to estimate total PO activity using the enzyme α-chymotrypsin to activate all pro-PO present in the haemolymph. We estimated total PO activity, instead of standing PO activity, for three reasons. First, levels of standing PO are undetectably low in *T. oceanicus* (N. Bailey, unpublished data). Second, pro-PO can be activated by a variety of proteases, including those contaminating glassware (Adamo, 2004a). Third, standing PO and total PO levels were found to be highly correlated in another cricket species (Adamo, 2004a).

We combined 5 μL of the PBS-bound hemolymph with 7 μL of bovine pancreas α-chymotrypsin (1.3 mg/mL, Sigma Aldrich C7762) and incubated the mixture for 20 min at room temperature (20 °C). We then added 90 μL of 15 mM L-dihydroxyphenylalanine (L-DOPA, ARCOS Organics AC167530050). PO converts L-DOPA to dopachrome, which we measured spectrophotometrically at 490 nm over 40 min during the linear phase of the reaction using a Bio-Rad 550 microplate reader. Total PO activity was estimated by measuring the change in absorbance over time due to the formation of dopachrome. Greater opacity of the solution indicated higher PO activity.

2.2.3. Lysozyme assay

Our lysozyme assay was adapted from Fedorka et al. (2004), Adamo (2004a) and Shoemaker et al. (2006). Lysozymes present in insect hemolymph are important in immune defense (Schneider, 1985) because of their role in degrading peptidoglycans in the cell walls of invading bacteria (Terra and Ferreira, 1994). We estimated LA turbidometrically by adding 10 μL of the PBS-bound hemolymph to a 0.35 mg/mL solution of the bacteria *Micrococcus luteus* (*lysodeikticus*) (powdered form, ICN Biomedicals 159972) and measuring the change in absorbance at 490 nm over 120 min using a Bio-Rad 550 microplate reader. In this case, greater transparency of the solution indicated higher LA.

2.3. Respiratory funnel dissection and measurement

We measured the degree of melanization of all the respiratory funnels in each cricket. Each funnel attaches to the abdominal wall at its narrow end, the first 2–3 mm of which is heavily pigmented. We dissected each melanized tip, dry-mounted it on a glass slide and calculated a darkness value using the same equipment, software and technique as for the implants.

We tested for a relationship between the average melanization of respiratory funnels and the strength of change in each immune parameter. Such a relationship is expected if resources normally involved in encapsulation and PO production are redistributed towards the development of larval respiratory funnels. However, no such relationship was expected for LA, because it is not involved in encapsulation or melanization. Finally, we tested for an effect of the number of larvae developing within a host on the melanization of the respiratory funnels those larvae constructed.

3. Results

3.1. Body size, age and immune response

Body size did not predict change in immune response in any of the assays (linear regressions: all $p > 0.201$) or absolute values of immune response (general MANOVAs: all $p > 0.080$), so we omitted it from subsequent analyses. Age did not predict changes in encapsulation or PO activity for both control or experimental crickets or LA for control crickets (linear regressions: all $p > 0.304$), but it predicted the change in LA in infested crickets (linear regression: r^2 adj. $= 10.9$, $p = 0.004$) so we included it as a covariate in the model examining LA. When testing the relationship between the change in LA and the number of larvae harbored, we did not include age as a covariate because there was no relationship between age and the number of larvae a cricket contained (Pearson correlation: $r = 0.077$, $n = 67$, NS).

3.2. Effect of infestation on immune responses

Table 1 gives results from separate repeated measures ANOVAs for each immune response measured. The time × treatment interaction term is of particular interest, because it indicates whether the magnitude of change in immune response between the first and second assays (time) differs for control and infested crickets (treatment).

Table 1
Results of three separate repeated measures ANOVAs examining the effects of time (pre-infestation versus post-infestation) and treatment (control versus infested) on encapsulation, PO activity and LA

	d.f.	*F*	*p*
Encapsulation			
Time	1	11.25	**<0.001**
Treatment	1	3.20	0.077
Time × treatment	1	0.28	0.282
Error	93		
PO activity			
Time	1	61.36	**<0.001**
Treatment	1	4.98	**0.028**
Time × treatment	1	3.16	0.079
Error	98		
LA			
Time	1	0.52	0.475
Treatment	1	18.22	**<0.001**
Time × treatment	1	15.52	**<0.001**
Age	1	32.98	**<0.001**
Time × age	1	8.73	**0.004**
Error	92		

The interaction term time × treatment tested the homogeneity of slopes between control and infested crickets. Age was only included as a covariate in the analysis with LA. Significant *p*-values are in bold.

Both control crickets and infested crickets showed significantly decreased encapsulation ability during the second assay (Fig. 1). Encapsulation ability in infested crickets showed a greater decrease than controls, but a non-significant time × treatment interaction term indicates the slope of change did not differ between the two groups (Fig. 1). Infested crickets showed a precipitous decline in LA over pre-infestation levels, but control crickets subjected to the same handling regime over the same time period showed no change in LA activity (Fig. 2). PO activity in both infested and control crickets was significantly elevated after the second assay (Fig. 3). Infested crickets showed a roughly 60% greater increase in PO activity than control crickets, and there was a marginally non-significant trend for PO activity in infested crickets to increase more rapidly over the 3-day period (Table 1).

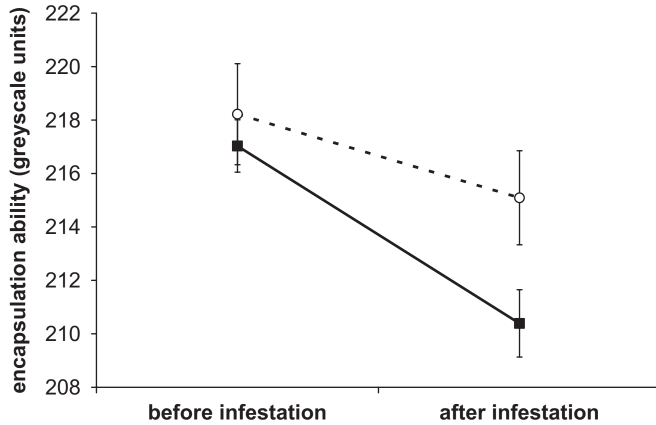

Fig. 1. Change in encapsulation ability in infested crickets ($n = 69$, solid line) and uninfested controls ($n = 29$, dashed line). Error bars indicate standard error. Both treatments showed a significant decrease in encapsulation ability 3 days after infestation, and the slope of change did not differ between them.

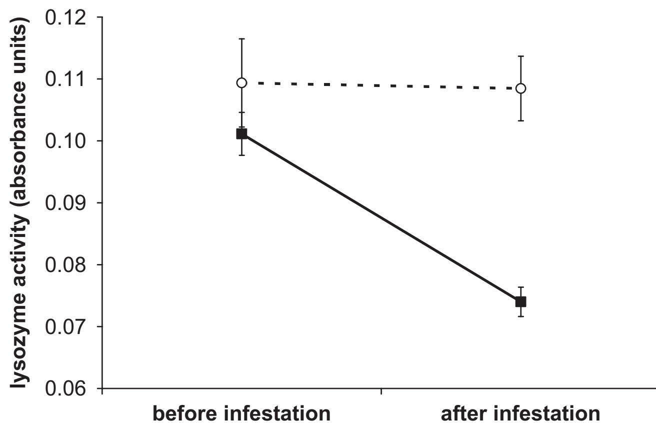

Fig. 2. Change in lysozyme activity (LA) in infested crickets ($n = 67$, solid line) and uninfested controls ($n = 27$, dashed line). Error bars indicate standard error. Infested crickets had significantly lower LA 3 days after infestation, whereas LA did not change in uninfested controls.

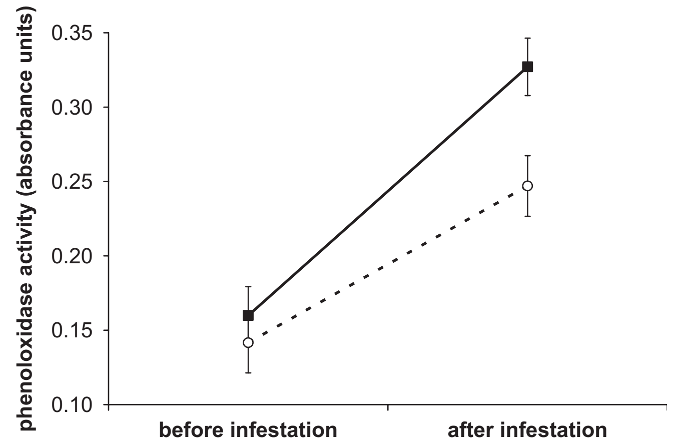

Fig. 3. Change in phenoloxidase (PO) activity in infested crickets ($n = 67$, solid line) and uninfested crickets ($n = 27$, dashed line). Error bars indicate standard error. Both groups had significantly higher PO activity 3 days after the initial challenge, although there was a marginally non-significant trend for PO activity to increase more rapidly in infested crickets.

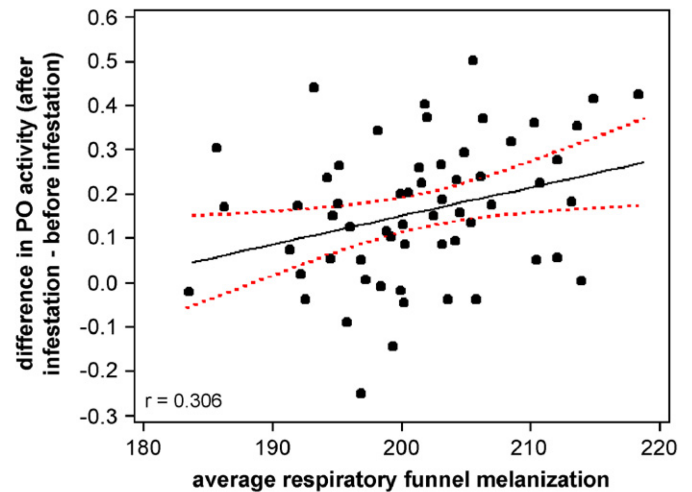

Fig. 4. Correlation between the change in host phenoloxidase (PO) activity and larval respiratory funnel melanization. Dashed lines indicate 95% confidence bands. Crickets that produced more PO after infestation harbored larvae with significantly darker, more heavily melanized respiratory funnels.

3.3. Respiratory funnel melanization

Crickets that produced more PO after infestation harbored larvae with darker, more heavily melanized respiratory funnels ($r = 0.306$, $n = 59$, $p = 0.018$) (Fig. 4). However, we found no relationship between change in crickets' encapsulation ability or LA after infestation and the degree of melanization of respiratory funnels of larvae growing inside of them ($r = -0.045$, $n = 60$, NS and $r = 0.061$, $n = 58$, NS; respectively). Also, there was no correlation between the number of fly larvae parasitizing a cricket and their average respiratory funnel melanization score ($r = -0.028$, $n = 60$, NS).

3.4. Relationship between immunity measures and number of larvae

The number of larvae developing within crickets was correlated with changes in encapsulation ability and LA of those crickets (Figs. 5 and 6). Crickets that harbored fewer larvae showed a greater decrease in encapsulation ability (r^2 adj. = 8.7, $F_{1,64} = 7.22$, $p = 0.009$). In contrast, crickets with more larvae showed a greater decrease in LA (r^2 adj. = 10.0, $F_{1,62} = 7.97$, $p = 0.006$). PO activity remained constant regardless of how many larvae a cricket contained (r^2 adj. = 0.1, $F_{1,63} = 0.09$, NS). It is noteworthy that four infested crickets contained no larvae when we dissected them. We included this small number of comparisons in the analysis because one interpretation is that these crickets managed to encapsulate and kill all three planidia; however, when we restricted the regression analysis to crickets that were found to contain only one, two or three larvae, the results remained the same for encapsulation, LA and PO (r^2 adj. = 8.2, $F_{1,62} = 6.63$, $p = 0.012$; r^2 adj. = 8.4, $F_{1,59} = 6.47$, $p = 0.014$; r^2 adj. = 0.0, $F_{1,60} = 0.00$, NS; respectively).

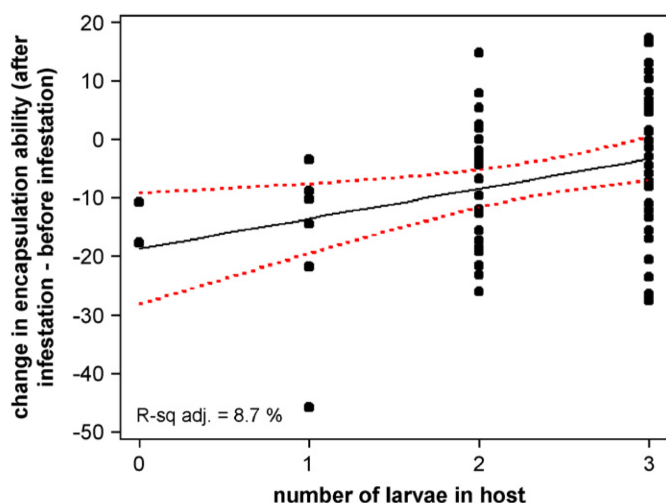

Fig. 5. Linear regression of the change in host encapsulation ability on number of larvae harbored. Dashed lines indicate 95% confidence bands. Crickets harboring fewer larvae showed a significantly greater decrease in encapsulation ability after infestation. When the apparent outlier at −45 in the group of 1 larvae per host was omitted, the regression was still significant.

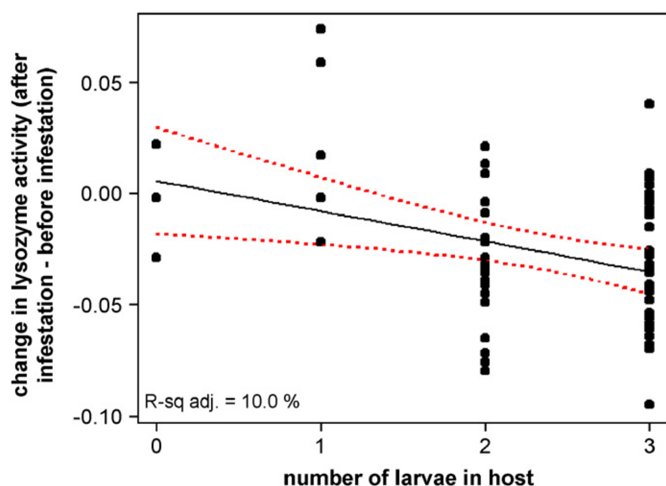

Fig. 6. Linear regression of the change in LA on number of larvae harbored. Dashed lines indicate 95% confidence bands. Crickets harboring more larvae showed a significantly greater decrease in LA after infestation.

4. Discussion

T. oceanicus hosts undergo substantial immunological changes when infested with mobile *O. ochracea* larvae. Additionally, pre-challenging crickets with nylon implants had an enduring effect on two immune parameters. Encapsulation ability decreased and PO activity increased for both control and infested crickets, whereas LA declined only in infested crickets and remained constant in control crickets. Control and infested crickets did not differ in the magnitude of change in encapsulation ability, but there was a marginally non-significant trend for infested crickets to increase PO activity more rapidly than controls. The decrease in encapsulation ability and increase in PO activity are likely typical responses in *T. oceanicus* to invasion by stationary particles such as hymenopteran eggs or the artificial nylon inserts we used in this study. However, fly larvae may continue to stimulate immunological responses as they locomote within and consume the interior of their host, accounting for the 60% higher PO activity of infested crickets compared to controls.

Each cricket was infested with equal numbers of planidia, but not all of them became established. Crickets harboring fewer larvae exhibited greater decreases in encapsulation ability, which is consistent with the hypothesis that decreases in encapsulation during the crucial time period when larvae begin co-opting the host response to construct respiratory funnels may contribute to more effective late control of parasitoid infestation. Decreased encapsulation ability during late larval infestation may provide crickets with a selective advantage in highly parasitized populations. Other evidence supports this. In a different study of *T. oceanicus*, consistently parasitized populations in Hawaii showed lower rates of encapsulation than unparasitized Australian populations (Zuk et al., 2004). It is also noteworthy that in our experiment, we observed three crickets that each contained one partially developed (late instar), but dead, larva. While anecdotal, these occurrences illustrate that late control of parasitoid infestation via interference with respiratory funnel construction may be possible, although we cannot exclude the possibility that the deaths resulted from intrinsic mortality or competition between larvae.

There is another plausible explanation for the observation that crickets harboring fewer larvae had lower encapsulation levels, however. Some hosts may have been

able to encapsulate invading planidia before they penetrated the abdominal wall. The results from the control group clearly show that an initial encapsulation challenge decreases the host's ability to mount a subsequent encapsulation response. We infected crickets with equal numbers of planidia, so hosts containing fewer third instar larvae may have had reduced encapsulation responses because they already encapsulated one or more planidia. Given the substantial and enduring effects of initial encapsulation challenge on all three immune parameters measured, it would be of interest to examine how the ability of crickets to control parasitoid infestation varies over the course of multiple encapsulation challenges. Our results suggest that host ability to encapsulate foreign invaders will likely decrease with each successive challenge.

In contrast, host PO activity increased over the course of the two assays in both treatment groups of this experiment. Moret and Siva-Jothy (2003) demonstrated that long-term increases in antimicrobial activity after an initial lipopolysaccharide (LPS) challenge conferred a future advantage to *Tenebrio molitor* infected with a fungal pathogen, but that PO activity did not show a similar increase after LPS challenge. In *T. oceanicus*, the initial encapsulation assay may have acted as a pre-challenge that induced a prophylactic upregulation of PO activity. The combined effect of the pre-challenge plus the immunological response to infestation by *O. ochracea* larvae likely accounts for the greater PO activity in the infested treatment versus the control treatment. It is noteworthy that PO activity was increased after the initial encapsulation challenge in this study, but that antimicrobial activity was increased after a initial LPS challenge in *T. molitor* (Moret and Siva-Jothy, 2003). The degree to which prophylactic responses are flexible according to the nature of the initial immune challenge warrants future investigation.

Surprisingly, infested crickets also showed substantial decreases in LA, with larger decreases occurring in crickets containing more larvae. This was a unique effect of harboring larvae, because control crickets similarly manipulated but left uninfested showed no change in LA. Lysozymes are involved in the antibacterial immune response in insects (Hoffmann et al., 1996, Gillespie et al., 1997), and LA was therefore predicted to remain unaffected by infestation. There are two possible explanations for this counterintuitive result. First, larvae may actively suppress LA in their hosts. The ability of parasitoids to manipulate many aspects of their hosts' physiology, including immune responses, is well-documented (Vinson, 1990; Schmid-Hempel, 2005) though these efforts can be costly (Kraaijeveld et al., 2001). Second, LA may trade-off with other host immune responses such as PO activity, as has been suggested in a variety of insects (Moret and Schmid-Hempel, 2001; Moret and Siva-Jothy, 2003; Adamo, 2004a; Cotter et al., 2004). PO activity in infested *T. oceanicus* was roughly 60% higher than that in control crickets, which is consistent with the idea that crickets reallocate immunity resources towards greater PO production at the expense of LA during infestation. Whether hosts achieve this independently, or their parasitoids control such a trade-off, cannot be conclusively determined from this study. However, parasitoid larvae may enjoy an advantage developing in a host with a greater PO response, because we found that greater PO activity corresponds to larvae having more darkly melanized respiratory funnels.

Many studies have assessed the immunological responses of hosts to their pathogens, but the changes induced by highly mobile *O. ochracea* larvae contrast with those found in insect studies using bacterial or immobile parasitoid challenges. For example, the cricket *Gryllus texensis* responded to bacterial challenge by increasing LA and PO activity, and greater increases in LA were associated with greater resistance to the bacterial pathogens *Serratia marcescens* and *Bacillus cereus* (Adamo, 2004a). In contrast, diamondback moths (*Plutella xylostella*) infested with parasitoid wasp eggs showed age-dependent decreases in LA and PO activity (Bae and Kim, 2004). The presence of parasitoid eggs in *P. xylostella* also dramatically reduced total hemocyte count and had secondary effects on phagocytic ability, which was interpreted as contributing to overall suppression of the immune system (Ibrahim and Kim, 2006). If a similar response occurs in *T. oceanicus*, it may contribute to the demise of *O. ochracea* larvae if it interferes with the construction of their respiratory funnels and sheaths. Finally, in *Drosophila*, parasitization by hymenopteran eggs induces the rapid differentiation of hemocytes into components involved in encapsulation, indicating an upregulation of the immune response (Lanot et al., 2001).

The timing of immunity assays is critical for understanding how an organism responds to infection. Changes in a particular immune response may reflect reallocation of immune effort within the organism, as opposed to a change in overall immune capability, and such reallocations may be temporally flexible during infection (Braude et al., 1999; Adamo, 2004b). Just prior to the emergence of *O. ochracea* larvae, both LA and PO activity in infested *T. oceanicus* crickets decrease to almost unmeasurably low levels (N. Bailey, unpublished data). PO activity thus increases initially before greatly decreasing as infestation with highly mobile larvae progresses, whereas LA remains consistently decreased throughout the latter half of the infestation. For the purpose of this study, we were interested in the crucial period during which larvae begin to construct respiratory funnels, but future investigations should seek to establish whether host changes in immune allocation over time reflect different immunological strategies depending on the stage of parasitoid larval development.

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