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RESEARCH ARTICLE

Seasonality influences cuticle melanization and immune defense in a cricket: support for a temperature-dependent immune investment hypothesis in insects

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SUMMARY

To improve thermoregulation in colder environments, insects are expected to darken their cuticles with melanin *via* the phenoloxidase cascade, a phenomenon predicted by the thermal melanin hypothesis. However, the phenoloxidase cascade also plays a significant role in insect immunity, leading to the additional hypothesis that the thermal environment indirectly shapes immune function *via* direct selection on cuticle color. Support for the latter hypothesis comes from the cricket *Allonemobius socius*, where cuticle darkness and immune-related phenoloxidase activity increase with latitude. However, thermal environments vary seasonally as well as geographically, suggesting that seasonal plasticity in immunity may also exist. Although seasonal fluctuations in vertebrate immune function are common (because of flux in breeding or resource abundance), seasonality in invertebrate immunity has not been widely explored. We addressed this possibility by rearing crickets in simulated summer and fall environments and assayed their cuticle color and immune function. Prior to estimating immunity, crickets were placed in a common environment to minimize metabolic rate differences. Individuals reared under fall-like conditions exhibited darker cuticles, greater phenoloxidase activity and greater resistance to the bacteria *Serratia marcescens*. These data support the hypothesis that changes in the thermal environment modify cuticle color, which indirectly shapes immune investment through pleiotropy. This hypothesis may represent a widespread mechanism governing immunity in numerous systems, considering that most insects operate in seasonally and geographically variable thermal environments.

Key words: temperature-dependent immunity, plasticity, seasonality, phenoloxidase, lytic, ecoimmunology.

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INTRODUCTION

Seasonal dynamics in pathogen infection rates have long been recognized (Nelson and Demas, 1996; Grassly and Fraser, 2008). These shifts may be due to stable cycles in vector abundance, pathogen infectivity or pathogen abundance outside of the host. Seasonal changes in host behavior (e.g. increased social contact or migration) and physiology may also play a significant role in infection rate dynamics (Altizer et al., 2006). This is most apparent during vertebrate breeding seasons, where potential hosts have been shown to increase physiological investment in reproduction at the expense of immunity, making them more susceptible to infection (Sheldon and Verhulst, 1996; Martin et al., 2008). Unfortunately, the majority of the data concerning seasonal fluctuations in immune function come from studies on birds and mammals, which represent long-lived organisms that tend to experience repeated annual breeding cycles. To date, no large-scale seasonal patterns in immune function have been identified in invertebrates. Unlike vertebrates, invertebrate systems tend to comprise short-lived adults that do not experience multiple breeding seasons, suggesting that seasonal immune changes are uncommon. Cyclical shifts in climactic conditions, however, may still have a profound impact on invertebrate immune function. Here we examine the hypothesis that seasonal shifts in the thermal environment alter an insect's thermoregulatory strategy, which indirectly alters immune investment.

Insects rely on external sources of heat to undertake basic physiological processes. As a consequence, insect cuticles in colder geographic locations are predicted to become replete with melanin, which darkens the cuticle and increases absorption of solar radiation [i.e. heat (Watt, 1968; Clusella Trullas et al., 2007)]. This prediction has been supported in several insect species, whose high latitude and/or altitude populations exhibit darker, more melanized cuticles (Majerus, 1998). Cuticle darkening is facilitated by the enzyme phenoloxidase (PO), which converts L-DOPA (or dopamine) to melanin (True, 2003). However, the same PO-driven process that darkens cuticle also helps to defend against infectious agents within the hemocoel (Cerenius and Söderhäll, 2004). When infected, host hemocytes agglutinate around an invader (e.g. bacteria, parasitoids or nematodes) and form a melanized capsule. As the capsule darkens, cytotoxic intermediates are produced that reduce the invading organism's ability to survive. The physiological link between cuticular melanin and melanin-based immune function in insects suggests that thermal selection on cuticle color should indirectly shape investment in melanin-based immunity, which we refer to as the 'temperature-dependent immune investment hypothesis' (Fedorka et al., 2013). A major prediction of our hypothesis is that the need for darker cuticles in colder environments will induce a general increase in melanin-producing infrastructure, which will endow the population with a more robust melanin-based immune defense.

Initial support for this hypothesis was found by examining nine thermally distinct populations of the ground cricket *Allonemobius socius* (Scudder). These populations were distributed along a 500 km latitudinal gradient within the eastern United States that varied in

the annual amount of thermal energy (i.e. degree days) available (Fedorka et al., 2013). We found that males and females from colder populations exhibited darker cuticles. As predicted by the temperature-dependent immune investment hypothesis, we found that colder populations also exhibited a greater degree of PO circulating in the hemolymph, which is predictive of a greater immune defensive ability. It should be noted that these patterns were not the result of plasticity, but of local adaptation, as each thermally distinct population was examined under common garden laboratory conditions. Thus, geographic differences in the thermal environment appear to shape population genetic differences in cuticular melanism and melanin-based immune function.

Of course thermal environments vary seasonally as well as geographically, which should select for a large degree of plasticity within populations for melanin investment. If true, then melanin-based immune ability should track seasonal thermal changes, with defense against infectious agents increasing as populations shift from summer into fall environments. Here we address the idea of temperature-dependent immune investment by examining the potential for seasonal shifts to induce plastic changes in cuticular melanin and pathogen resistance. To this end we chose a previously examined *A. socius* population that exhibits a bivoltine breeding season (two generations per year) under natural conditions, with the first generation experiencing a warm summer thermal environment while the second generation experiences a cool fall thermal environment. We expected plasticity in cuticle darkness to exist in this population, assuming a need to optimize cuticle color within each generation. As a consequence, we expected the cooler fall environment to induce a more robust melanin-based immune function compared with the warmer summer environment.

MATERIALS AND METHODS

Study system and temperature regimes

Allonemobius socius is a small chirping ground cricket found throughout the southeastern United States, from Florida to West Virginia and as far west as Texas (Traylor et al., 2008). In the northern part of its range (above 36°N latitude), *A. socius* is univoltine, producing one generation per year (Fedorka et al., 2012). In the southern part of its range (below 36°N latitude), *A. socius* is bivoltine or even multivoltine. Thus, the southern populations experience dramatic shifts in their thermal environment from generation to generation within a given year. To determine whether such thermal shifts maintain plasticity in cuticle color and immune function, we examined a southern bivoltine population collected from South Carolina (34°44′N 81°15′W). This laboratory population was derived from ~175 adults caught in August 2007 and maintained in incubators (Percival, Perry, IA, USA) at 28°C with a 9 h:15 h light:dark photoperiod. Under these conditions, female crickets lay diapause eggs that must overwinter at 4°C for several months prior hatching. In 2010 (after approximately five generations) the incubator environment was changed to a 28°C with a 14 h:10 h light:dark photoperiod to create a non-diapause environment and allow the production of continuous generations. All crickets were maintained in plastic boxes and provided with egg cartons for cover, as well as ground cat food and carrots for sustenance. Water-soaked cheesecloth was used as a source of water and for oviposition material. Cages were changed every other day.

To address our hypothesis, newly hatched individuals were randomly assigned to either a summer environment (day temperature: 28.5°C; night temperature: 26.5°C; 14 h:10 h light:dark photoperiod) or a fall environment (day temperature: 23.5°C; night temperature: 21.5°C; 11 h:13 h light:dark photoperiod) in the fall of 2011. These artificial environments are similar to the temperature and photoperiod experienced by the first (July) and subsequent (September–October) generations at the population's site of origin. Photoperiod was varied along with temperature because photoperiod has been shown to be a reliable cue for predicting seasonal changes in temperature (Bradford and Roff, 1993). Within each temperature regime, males and females were separated upon adult ecolsion to maintain virginity. To minimize the influence of temperature on metabolic rate and enzymatic activity, all crickets were moved at 10 days post eclosion into a common environment (day temperature: 26°C; night temperature: 24°C; 12 h:12 h light:dark photoperiod) for 24 h prior to having their immune function assayed.

Immunological assessment

Once in the common environment for 24 h, crickets were randomly assigned to one of two immune assay groups, which included a hemolymph collection group and a bacterial challenge group. In the hemolymph collection group, we employed two different immunological measures, including the PO and lytic assay. These assays provide an assessment of potential immune defense and are based on the concentration of constitutively expressed PO and lysozyme-like enzymes circulating in the hemolymph. To collect hemolymph, crickets were anesthetized on ice for 4 min and 2 µl of hemolymph were removed from between the second and third abdominal sternites using a microsyringe (Hamilton, Reno, NV, USA). Hemolymph was then expelled into a chilled microcentrifuge tube containing 20 µl of phosphate buffered saline (PBS) and frozen at –80°C. Each hemolymph sample was used to estimate an individual's PO and lytic activity, and to provide an estimate of total protein concentration. After hemolymph removal, crickets were then measured for the degree to which their cuticles were melanized (see Morphological measures, below).

To estimate PO activity, 4 µl from each sample was added to a separate well of a flat-bottom 96-well plate. We then added 14 µl of bovine pancreas α-chymotrypsin (Sigma-Aldrich, St Louis, MO, USA; 1.3 mg ml^{-1} solution) and the plate was incubated for 20 min at room temperature (α-chymotrypsin catalyzes the conversion of inactive PO into its active form, allowing all hemolymph-bound PO to be used in the analysis). After incubation, 90 µl of 7 mmol l^{-1} L-DOPA (Sigma-Aldrich) was added to each well using a multichannel pipette. Samples were randomly placed on the plate relative to treatment and sex. PO activity was estimated with a microplate reader (model 680, BioRad Laboratories, Hercules, CA, USA) as a total change in absorbance over 30 min at 490 nm (A_{490}). As the PO reaction proceeds, absorbance increases because of the formation of melanin. Those individuals with the greatest amount of circulating PO are expected to exhibit the greatest change in absorbance (ΔA_{490}). Previous work in our laboratory has found this estimate to be highly repeatable within individuals, and the reaction is linear over 60 min (K.M.F., unpublished data).

To estimate lytic activity, 16 µl of each sample was likewise dispensed into a flat-bottom 96-well plate, to which 90 µl of a *Micrococcus luteus* solution (0.003 g 1 ml^{-1} PBS) was added. As with PO, lytic activity was estimated as the change in absorbance over 30 min at 450 nm. In contrast to the PO reaction, absorbance decreases as the reaction proceeds because of the lysing of bacterial cell walls. Similar to the PO reaction, however, individuals with the greatest activity are expected to exhibit the greatest change in absorbance (ΔA_{450}). To avoid confusion, the lytic ΔA_{450} was rendered positive by taking the absolute value of the estimate. Thus, large positive values of ΔA_{450} indicate a greater lysozyme-like activity. To estimate protein concentration, 1 µl of each sample was

added to 99 µl of Bradford reagent (Thermo Scientific, Town, State, Country) and dispensed into a 96-well plate. After an 8 min incubation period at room temperature, a single absorbance reading at 595 nm was obtained. Protein concentration was estimated *via* a BSA standard (Thermo Scientific).

In the bacterial challenge group, individuals were inoculated with an LD_{50} of *Serratia marcescens*. To obtain the LD_{50}, bacteria were reared in sterile broth at 37°C for 18 h, at which point a subsample was diluted down to an A_{490} of 0.1 using a microplate reader. This was followed by an additional dilution to 1.5×10^{-2} of the 0.1 A_{490} concentration. A 1 µl dose of this concentration was injected between the second and third abdominal sternites using a microsyringe. Once injected, crickets were housed in individual Petri dishes (5.5 cm diameter) containing food and water for 72 h and survival was assayed [previous work suggests that after 72 h, infected individuals have either died or recovered (citation??)]. In contrast to the hemolymph assays, the bacterial challenge assay provides an assessment of realized immune defense.

Morphological measures

Crickets from the hemolymph assay were also used to estimate the degree to which the cuticles were melanized. For each individual, we captured four images using a digital camera (Dino-Lite, Taiwan) mounted on a dissecting microscope (Leica Model G26, Town, State, Country). These were top-down images of the pronotum, left femur, abdomen (with wings removed) and wings (removed and mounted to a microscope slide). The degree of melanism was measured as the mean grey scale darkness of the pixels, with 0 being completely white and 255 being completely dark, using ImageJ software (http://imagej.nih.gov). To control for subtle differences in lighting between images, we calculated the average of two control areas: one at the top and bottom of each image. The control darkness was then subtracted from the cuticle darkness. Together, the body parts assayed accounted for ~85% of an individual's dorsal area exposed to direct sunlight (excluding the head, prothoracic legs and mesothroacic legs; exposure area estimated *via* a top-down image using ImageJ).

Importantly, darker cuticles are expected in larger individuals because of their smaller surface area to volume ratio (assuming thermal inertia is not of concern). As each standard area of cuticle is responsible for heating more mass in larger individuals, a darker cuticle would help larger individuals keep metabolic pace with smaller conspecific competitors. The need for darker cuticles in colder environments may be negated if these individuals are smaller than their warmer temperature counterparts (increases surface to volume ratio). Previous work in *A. socius* has indeed shown that populations from colder geographic regions tend to be smaller (Fedorka et al., 2013), indicating the importance of including body size into our analyses. To this end, body size was estimated as femur length using ImageJ for both the hemolymph group and the bacterial challenge group.

Statistical analyses

In total, 173 individuals were used in the hemolymph assay, while 95 individuals were used in the bacterial challenge assay. To address the hypothesis that the environment influences immune function and cuticular melanization, we constructed several ANCOVA models. Each ANCOVA examined a separate response variable (cuticular melanization, PO activity, lytic activity, etc.) with environment (summer or fall), sex and wing morphology as fixed effects and femur length as a covariate. For each model, we report the standardized slope of the relationship (β) between the response variable and each of the independent variables. This model parameter provides the magnitude and direction of the relationship after controlling for other model variables. With regard to wing morphology, *A. socius* eclose as either a flight-capable morph (macropterous) or a flight-incapable morph (micropterous). Wing morphology was included to address the additional hypothesis that flight-capable macropterous morphologies may eclose darker in order to gain heat more quickly for prolonged flight. In all ANCOVA models, the interaction terms were discarded if they were not significant and the model reanalyzed. All analyses were conducted in JMP version 9 (SAS Institute, Cary, NC, USA).

RESULTS

As with previous work (Fedorka et al., 2007), we found that females were larger than males (controlling for environment, femur length was 5.7±0.04 mm *versus* 6.1±0.05 mm, respectively; $F_{1,171}=41.7$, $P<0.0001$), and the summer-like environment produced larger individuals relative to the fall-like environment (controlling for sex, femur length was 6.3±0.04 mm *versus* 5.4±0.05 mm, respectively; $F_{1,171}=200.6$, $P<0.0001$). We also found a greater proportion of flight-capable macropterous phenotypes in the summer *versus* the fall environment (27.1% *versus* 4.3%, respectively; $\chi^2=17.18$, $P<0.0001$). This pattern implies that the majority of gene flow between populations occurs in the summer months when the extended breeding season allows for both migration and reproduction. No difference in wing morphology between the sexes was detected.

Abdomen, femur and pronotum darkness were all positively correlated with one another (all $r>0.81$, all $P<0.0001$), while wing darkness was positively correlated with only femur darkness ($r=0.15$, $P=0.0277$). To simplify the analysis, all four measures of darkness were combined into a single synthetic factor *via* a principle component analysis. Subsequent analyses used the first principle component as a measure of overall cuticular melanization, which explained 68% of the variation among variables. With regard to the immune parameters, PO activity was positively correlated with lytic activity after controlling for sex, wing morphology and environment (β=0.45, $F_{1,167}=36.7$, $P<0.0001$). In addition, hemolymph protein concentration was positively associated with lytic activity (β=0.19, $F_{1,131}=4.51$, $P=0.0357$). Thus, individuals with a strong lytic response also tended to have a strong PO response (Fig. 1) and a greater amount of circulating proteins. We found no association between overall cuticular melanization and potential immunity within environments (after controlling for sex and wing morphology).

With regard to the temperature-dependent immune investment hypothesis, we found that the thermal environment had a significant influence on overall cuticular melanization (Table 1), with darker cuticles found in the fall-like environment and among males (Fig. 2). Moreover, thermal treatment had a significant influence on all three hemolymph measures (Table 1), with the fall-like environment possessing a greater PO activity as predicted (Fig. 2). However, this environment also induced a weaker lytic activity, which suggests a cost to increasing melanin-producing infrastructure (Fig. 1). Despite the weaker lytic estimate, the bacterial challenge assay indicated that the fall-environment individuals were better able to defend against a live pathogen in a common environment (Table 2, Fig. 2). These data imply that the PO assay may serve as a superior predictor of immune defense. To further investigate the interaction between body size and wing morph in the lytic model, the wing morphs were analyzed separately. We found that that the interaction was driven by a positive relationship between body size and lytic activity in

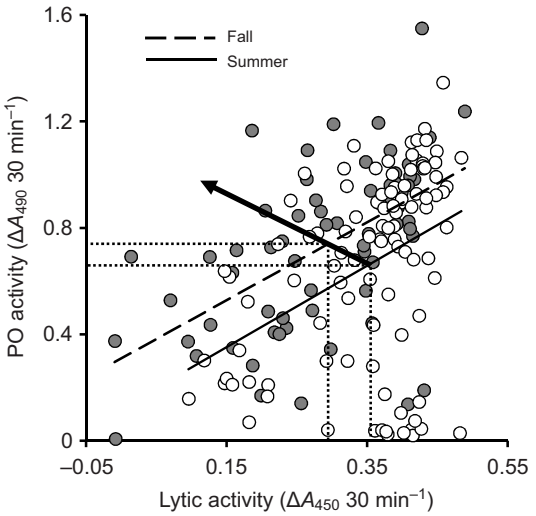

Fig. 1. Relationship between lytic and phenoloxidase (PO) activity within and across environments in the cricket *Allonemobius socius*. A large change in absorbance indicates a strong PO activity and a strong lytic activity. Thus, these activities are positively correlated within environments. Interestingly, the fall-like environment (dashed least squares regression line, filled circles) exhibited a greater average PO activity but a weaker average lytic activity compared with the summer-like environment (solid least squares regression line, open circles), suggesting a cost to increased melanin-based immunity in the fall environment. Dotted lines highlight the average PO and lytic activities in both environments and the arrow represents the directional change in immunity from summer to fall.

Table 1. Role of environment on melanization and immunity: melanization and hemolymph assays

Source	d.f.	*F*	*P*	β
Response=cuticular melanization				
Model	4,155	16.6	**<0.001**	
Environment (fall)	1,158	4.6	**0.0335**	0.23
Sex (female)	1,158	11.1	**0.0011**	−0.27
WM (short)	1,158	4.2	**0.0433**	−0.16
BS	1,158	0.5	0.4916	−0.08
Response=total PO				
Model	4,163	3.6	**0.0074**	
Environment (fall)	1,166	4.2	**0.0411**	0.23
Sex (female)	1,166	12	**0.0007**	−0.29
WM (short)	1,166	0.3	0.5804	0.04
BS	1,166	2.4	0.1267	0.18
Response=lytic activity				
Model	5,161	6.9	**<0.001**	
Environment (fall)	1,165	8.3	**0.0045**	−0.31
Sex (female)	1,165	4.6	**0.0343**	−0.17
WM (short)	1,165	0.01	0.9204	−0.01
BS	1,165	3.4	**0.0675**	−0.26
WM×BS	1,165	7.7	**0.0062**	0.32
Response=protein				
Model	4,128	6.6	**<0.001**	
Environment (fall)	1,131	7.1	**0.0089**	−0.32
Sex (female)	1,131	0.8	0.3687	−0.08
WM (short)	1,131	1.1	0.2937	0.09
BS	1,131	1.2	0.2736	0.14

WM, XXXX; BS, XXXX.
Bold indicates XXXXX.

the macropterous morph, but no such relationship existed in the micropterous morph. Last, the macropterous morph was darker than the micropterous morph (0.67±0.31 *versus* –0.02±0.14, respectively; Table 1), which may be an adaptation that improves flight capability by allowing the absorption of more thermal energy. However, no difference in immune defense was found between the two morphs (Tables 1,2).

DISCUSSION

Here we show that seasonal changes in the environment (mimicked *via* changes in incubator temperature and photoperiod) had a profound influence on cuticle color and immune defense in this cricket system. Specifically, individuals from the cooler, fall-like environment exhibited a darker cuticle, a greater PO activity and a greater resistance to *S. marcescens*. Although the strength of the PO and lytic responses were positively correlated within environments, these responses appeared to trade off across environments, considering that fall-like individuals exhibited a stronger PO response and weaker lytic response compared with the summer-like individuals. This trade-off represents a potential cost of increased melanin-based investment. Despite this cost, fall-like individuals were better able to defend against a live pathogen, suggesting that additional costs to improved immune defense are likely to exist. In short, these data indicate that seasonal shifts in the thermal environment modify immune ability, as predicted by our hypothesis.

The changes in host physiology documented here are likely governed by the host's sensitivity to photoperiod and not necessarily to temperature. For instance, manipulation of photoperiod in vertebrate systems invariably alters immune function under laboratory conditions (Nelson and Demas, 1996). Photoperiod-driven circadian cycles have also been shown to influence invertebrate immune function (Stone et al., 2012; Watthanasurorot et al., 2013), with PO activity in the crayfish brain peaking in the early morning hours (Noonin et al., 2013). On a side note, it may be that these daily cycles in PO activity underlie the deposition of daily cuticle growth rings found in some arthropods. Regardless, it is important to note that photoperiod is the most reliable cue any organism has in predicting cyclical changes in the thermal environment (Nelson and Demas, 1996; Nelson, 2004). Therefore, in order to adapt to seasonal changes in temperature, we expect natural selection to favor individuals highly sensitive to shifts in day length, not temperature. With regard to *A. socius*, it would still be of interest to determine whether a similar effect can be obtained using temperature (or photoperiod) alone. It would also be of interest to examine parasite load in wild summer and fall generations to determine whether the shift in immune function uncovered here has a realized consequence under natural conditions.

As stated earlier, the adult stage in most insect systems tends to be ephemeral and does not span multiple seasons. However, some species are relatively long lived, and can overwinter as adults in cold climates by entering a state of diapause. If the acquisition of solar thermal energy is crucial to winter survival, then we would expect an increased investment in melanin-producing infrastructure to darken the cuticle, which would indirectly improve immune function. In the water strider *Aquarius naja*, males who were successful in surviving winter diapause also exhibited a greater melanin-based immune defense when confronted with a novel pathogen [a piece of nylon filament (Krams et al., 2011)]. Although the authors did not examine male cuticle color, these data are consistent with our hypothesis. Interestingly, cold environments appear to suppress immune defense in the cricket *Gryllus texensis* (Adamo and Lovett, 2011). Specifically, crickets maintained at 18 and 26°C exhibited a reduced PO activity and survival against an

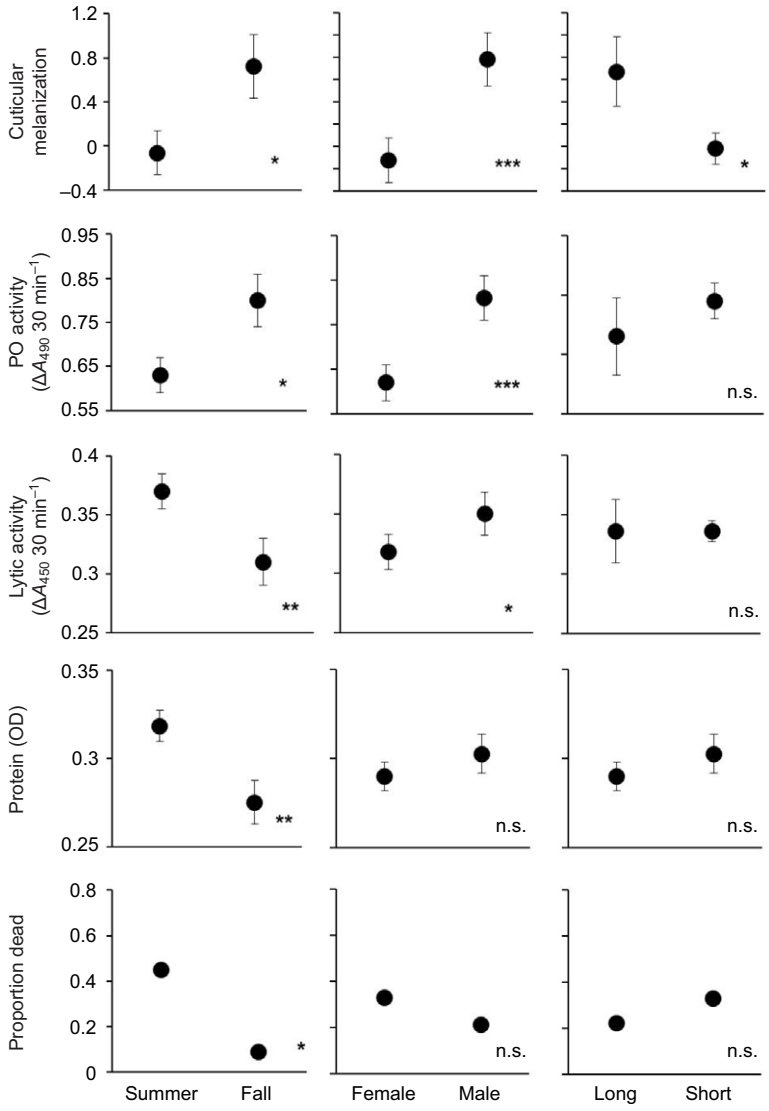

Fig. 2. Role of environment, sex and wing morphology on cuticular melanization and immune function in the cricket *Allonemobius socius*. Circles represent least squares mean and associated standard error, which were estimated using the models reported in Table 1. Asterisks denote statistical significance (*P<0.05, **P<0.01, ***P<0.005); n.s., not significant.

LD_{50} of *Seratia marcescens* compared with those maintained at 33°C. Although these data appear contradictory, all immune defense estimates in the *G. texensis* study were performed within the treatment temperatures, suggesting that decreased defensive capability at colder temperatures was the result of a reduced metabolic rate/enzymatic activity (further supported by decreased weight gain and oviposition rate in these treatments). In contrast, our immune measures were all carried out in a common environment, suggesting that differences in immune defense were not due to differences in metabolic rate, but to resources differentially allocated to melanin-producing physiology.

In accordance with previous work (Fedorka et al., 2013), we found that *A. socius* males were smaller and darker than females. However, the thermal melanin hypothesis predicts that smaller individuals should be lighter as a result of a higher surface area to volume ratio (Clusella Trullas et al., 2007). What maintains this counterintuitive dimorphic pattern is currently unknown, but it may be due to additional selection on males for darker cuticles. In this system, males tend to call most at dawn and dusk. Darker males may heat up faster, or store more thermal energy, than lighter males. This would allow them to engage in reproductive activities earlier in the day, or for a longer period at night. Darker males may also be able to call at much faster rates, which previous work has shown to be attractive to *A. socius* females (Fedorka and Mousseau, 2007; Olvido et al., 2010). In addition to improved reproductive energetics, darker males may be preferred by females because of their improved immune capacity [i.e. parasite-mediated sexual selection (Hamilton and Zuk, 1982)], causing cuticle to darken because of its strong correlation with melanin-based immunity. Accordingly, female choice for greater male immune function *via* melanized cuticle has been shown previously in some insect systems (Rantala et al., 2000).

The observation that darker cuticle color is correlated with a more robust melanin-based immune function in insects is not novel (Wilson et al., 2001). Previous work in the desert locust *Schistocerca gregaria* found that when reared under crowded conditions, individuals exhibited darker cuticles (typical of the gregarious phase) and increased resistance to the entomopathogenic fungus *Metarhizium anisopliae* (Wilson et al., 2002). Similar patterns have been found with other phase polyphenic insects that exhibit high-density forms (Barnes and Siva-Jothy, 2000; Cotter et al., 2004). These patterns are predicted by the density-dependent prophylaxis hypothesis, which states that crowding increases disease transmission risk, which induces increased investment in immune function (Wilson and Reeson, 1998). Importantly, this hypothesis predicts direct selection on immune function and not necessarily cuticle color, as melanin embedded within the cuticle provides no

Table 2. Role of environment on melanization and immunity: bacterial challenge assay

Source	d.f.	X^2	*P*
Response=dead or alive			
Model	4	7.61	0.1071
Environment (fall)	1	5.61	**0.0179**
Sex (female)	1	1.97	0.1602
WM (short)	1	0.76	0.3835
BS	1	3.96	**0.0465**

Bold indicates XXXX.

active immunological function (other than passive cuticle strengthening). The darker cuticles in these studies are likely a byproduct of the increased melanin-producing infrastructure needed for increased immunity. Our hypothesis is different in that we suggest that seasonally or geographically distinct thermal environments directly shape cuticle color, which indirectly shapes immune function through pleiotropy. Like density-dependent prophylaxis, our hypothesis may be a widespread mechanism governing numerous insect systems, considering the need for all insects to maximize fitness in a variable thermal environment.

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AUTHOR CONTRIBUTIONS

XXXXX

COMPETING INTERESTS

No competing interests declared.

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