Title: Thermoregulatory strategy may shape immune investment in *Drosophila melanogaster*

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ABSTRACT

As temperatures change, insects alter the amount of melanin in their cuticle to improve thermoregulation. However, melanin is also central to insect immunity, suggesting that thermoregulatory strategy may indirectly impact immune defense by altering the abundance of melanin pathway components (a hypothesis we refer to as thermoregulatory-dependent immune investment). This may be the case in the cricket *Allonemobius socius*, were warm environments (both seasonal and geographical) produced crickets with lighter cuticles and increased pathogen susceptibility. Unfortunately, the potential for thermoregulatory strategy to influence insect immunity has not been widely explored. Here we address the relationships between temperature, thermoregulatory strategy and immunity in the fruit fly *Drosophila melanogaster*. To this end, flies from two separate Canadian populations were reared in either a summer or fall-like environment. Shortly after adult eclosion, flies were moved to a common environment where their cuticle color and susceptibility to a bacterial pathogen (*Pseudomonas aeruginosa)* were measured. As with *A. socius*, individuals from summer-like environments exhibited lighter cuticles and increased pathogen susceptibility, suggesting that the thermoregulatory-immunity relationship is evolutionarily conserved across the hemimetabolous and holometabolous clades. If global temperatures continue to rise as expected, then thermoregulation might play an important role in host infection and mortality rates in systems that provide critical ecosystem services (e.g. pollination), or influence the prevalence of insect-vectored disease (e.g. malaria).

Insects are ectothermic poikilotherms whose body temperature covaries with ambient temperature. Still, insects can maintain some degree of independence from ambient temperature by employing a variety of thermoregulatory strategies (Heinrich, 1974); one of the most common of which is solar basking (May, 1979). Basking is commonly observed in the pre-flight warm-up of winged insects (e.g. butterflies) allowing them to quickly achieve the threshold muscle temperatures necessary for flight (Watt, 1968). However, non-flying insects also bask (e.g. ants; Frouz, 2000). Importantly, basking efficiency is increased through darker cuticles (Watt, 1968; Kingsolver, 1983). As one might expect, variation in thermal environments (geographic and seasonal) has been shown to induce variation (genetic and plastic) in cuticular melanin, with cooler environments producing darker individuals (Majerus, 1998; Clusella Trullas et al., 2007; Fedorka et al., 2013a; Fedorka et al., 2013b). In short, cuticular melanin appears to be under strong thermoregulatory selection in many species.

However, melanin is also a crucial component in insect immunity. When an insect host is confronted with a hemocoel-bound pathogen, it encapsulates the invading organism with hemocytes. The hemocytes degranulate to release a tyrosinase phenoloxidase (PO), which converts tyrosine to L-DOPA and several other diphenol quinones (Fig.1). These substrates are eventually transformed into melanochrome, which non-enzymatically converts to melanin. The reactive quinone intermediates produced during this biochemical cascade possess strong antibiotic properties, protecting the host from a wide range of pathogens (including bacteria, fungi, animals and viruses; Cerenius and Soderhall, 2004; Zhao et al., 2007; Cerenius et al., 2010; Gonzalez-Santoyo and Cordoba-Aguilar, 2012). Interestingly, the biochemical pathway that produces immune-related melanin is essentially the same pathway involved in cuticular melanization; both produce the same substrate intermediates and utilize many of the same enzymes (Fig. 1; Arakane et al., 2009). Such extensive pathway overlap suggests that modification of one phenotype (e.g. cuticle color) will influence the expression of the other phenotype (e.g. immunity). This has been clearly shown in *Tenebrio molitor*, where artificial selection for cuticle color altered melanin-based immunity (Armitage and Siva-Jothy, 2005), indicating that these traits are pleiotropically linked (Rolff et al., 2005).

Given that cuticle melanin seems to be under strong thermal selection and genetically correlated with melanin-based immunity, thermoregulatory strategy has the potential to indirectly shape immune defense in insects. This may be the case in the cricket *Allonemobius socius* (Scudder), where natural selection for cuticle color along a thermal gradient appears to have altered melanin-based immune function (Fedorka et al., 2013b). In this species, individuals from geographically warmer populations possessed lighter cuticles and reduced melanin-based immunity compared with their cooler population counterparts, and this pattern persisted when the populations were laboratory reared under common garden conditions. Seasonal changes in the thermal environment also appear to alter cuticle color and

immunity in this species by creating lighter, less immune competent individuals under warm summer conditions compared with cool fall conditions (Fedorka et al., 2013a). Thus, geographical and seasonal changes in the thermal environment appear to adaptively shape cuticle color, while non-adaptively altering immune defense in *A. socius*. If the influence of thermoregulatory strategy on immune investment is evolutionarily conserved across insects, then thermoregulation may act as a common immune constraint for insects in warm climates. Unfortunately, the potential for thermoregulatory-dependent immune investment (TDII) has not been widely explored.

There are of course instances in which the thermoregulatory-immunity phenomenon may be weak or non-existent. This is likely to occur in systems with strong non-thermoregulatory selection on the cuticle. For instance, if cuticle color is governed largely by crypsis, aposematism, mimicry or sexual selection, then thermal variation may have a reduced impact on the cuticle phenotype and hence, melanin-based immunity. Likewise, subterranean insects that rarely forage outside of their nest (e.g. termites) would derive little thermoregulatory benefit from a melanized cuticle. Even relatively simple traits like body size could influence the potential for TDII. Smaller organisms gain heat more quickly than larger ones due to a greater surface to volume ratio (Clusella Trullas et al., 2007). Thus, species of smaller size may exhibit little variation in cuticular melanization across thermal environments due to their increased heat-capturing efficiency. A poor correlation between juvenile and adult thermal environments may also limit the potential for TDII, considering that much of the investment in cuticular melanin occurs during the adult molt. This may be particularly true in holometabolous insects whose larvae tend to develop in separate environments from the adults (e.g. fruit flies developing within a food patch); especially when the larval environment is distributed across highly variable microhabitats (e.g. fruit flies developing within fruit exposed to direct sunlight versus shade). Unfortunately, we do not know if any of the above organismal attributes limit the potential for TDII.

In this study, we address the potential for thermoregulatory-dependent immune investment in the fruit fly *Drosophila melanogaster* (Meigen 1830). The current system differs from *A. socius* in several key ways that may impact the potential for TDII. First, *D. melanogaster* are part of the holometabolous insect clade, which diverged from hemimetabolous insects in the Carboniferous period approximately 350 million years ago (Weinstock et al., 2006). As a consequence, the correlation between larval and adult thermal environments in *D. melanogaster* is expected to be weak compared to *A. socius*, whose hemimetabolous juveniles occupy similar microhabitats as adults. In addition, *D. melanogaster* are dramatically smaller than *A. socius* (~1mg versus ~100mg, respectively), which may make them more sensitive to shifts in the thermal environment. Both of these organismal attributes could alter the influence of the thermal environment on cuticle color, and hence immune function. We address the potential for TDII in two populations of *D. melanogaster* by rearing individuals in either a summer or fall-like

environment. Upon adulthood, individuals were moved to a common environment where their cuticle color and immune function were assayed. If temperature is found to influence cuticle color and immune defense in *D. melanogaster*, then TDII may be a fundamental aspect of insect physiology.

RESULTS

Consistent with most insect systems, females were larger than males (Table 1; mean ± s.e.m.: 0.84 ± 0.005 mm versus 0.72 ± 0.005 mm, respectively) and a small size difference was detected between the two populations examined (0.71 ± 0.006 mm versus 0.69 ± 0.006 mm for MO and SO, respectively). Importantly, flies from the fall environment exhibited a larger pronotum length than those form the summer environment (0.81 ± 0.005 mm versus 0.76 ± 0.005 mm, respectively), creating the potential for type I error when assessing the influence of environment on cuticle darkness (to improve thermoregulation, larger flies are expected to possess darker cuticles due to their smaller surface to volume ratio; Clusella Trullas et al. 2007). To minimize this potential we included size in our test of environmental treatment on cuticle color.

Pronotum color was strongly correlated with abdomen color after controlling for treatment (summer or fall), population (MO or SO), sex, pronotum length and incubator ($\beta = 0.65$, $P < 0.0001$). To create a composite estimate of cuticle darkness, the first principle component for these two measures was generated, which explained 74% of the variance. We found that environmental treatment had a significant influence on the degree of darkness (Table 1; Fig. 2). Although the extent to which the populations responded to the environmental treatment differed (creating a population by environment interaction), fall flies consistently exhibited darker cuticles. In addition to their darker cuticles, fall-reared flies were significantly more resistant to bacterial infection than were their summer counterparts (Table 1; Fig. 2), with 75% more summer-reared flies dying after 72 hours.

DISCUSSION

The thermal melanin hypothesis predicts that ectotherms of large size or from cold environments should exhibit darker cuticles compared with their warm environment counterparts (Clusella Trullas et al., 2007). Evidence for this hypothesis comes from several insect systems that vary in cuticular melanization across altitudinal and latitudinal gradients (Majerus, 1998). Here we show that flies reared in a cooler, fall-like environment are indeed darker than their warm environment counterparts; even after controlling for significant differences in body size across the environments. Logical extension of thermal melanin hypothesis suggests that thermal modification of cuticle color could influence melanin-based immunity due to their presumed genetic correlation (e.g. Fig. 1), which we refer to as the thermoregulatory-dependent immune investment hypothesis (TDII). In support of this hypothesis, we found that flies reared

in a summer-like environment were not only lighter in color, but also less resistant to infection with the gram-negative bacterium *Pseudomonas aeruginosa* (infected under standardized conditions). This pattern is consistent with direct thermal selection on cuticular color, coupled with indirect selection on melanin-based immunity.

Our current study, coupled with our previous work in the cricket *A. socius* (Fedorka et al., 2013a; Fedorka et al., 2013b), suggests that TDII may be ubiquitous in insects, except perhaps when alternative forms of intense selection on the cuticle color exist (e.g. aposematism, sexual selection, crypsis, etc). Considering that reduced immunity generally leads to increased infection rates, TDII should constrain host fitness or abundance in warm environments. This may cause significant disruption in systems where insects provide important ecosystem services (e.g. pollinators). Moreover, if a pathogen is vectored by an insect host, and the host mounts a reduced immune response against the pathogen, then the prevalence of the infection in the definitive host species may also increase with temperature. For example, the incidence of malaria may increase in warmer environments in part due to a weakened *Anopheles* mosquito immune system, which allows more *Plasmodium* to be transmitted. Accordingly, TDII could play an important role in the prevalence of insect-vectored human disease as global mean temperatures continue to rise.

As stated earlier, temperature is positively correlated with insect metabolic rates, which can allow for an improved immune response at higher temperatures (Adamo and Lovett, 2011; Catalan et al., 2012). This is clearly the case in *Tenebrio molitor*, where higher temperature induced a higher standing metabolic rate, greater antibacterial defense and greater phenoloxidase activity (Catalan et al., 2012). At first glance, this pattern implies climate change will tend to improve insect immunity. However, this type of experiment is generally conducted using organisms reared under similar conditions and then allocated to their temperature treatments after adult eclosion. Our data imply that as rearing temperatures increase (which is expected under climate change), insects reduce their investment in cuticular melanin, which may cause an overall decline in melanin-based immunity (Figure 3). However, to determine the impact climate change may have on insect immune defense, the relationships between rearing temperature, ambient infection temperature and immune function must be fully explored.

Considering that cuticle melanin and melanin-based immunity appear to be genetically correlated, the most likely candidate genes underlying this correlation are those involved in the melanin pathways (Fig. 1). The starting material (i.e. phenylalanine / tyrosine), substrate intermediates and most of the major enzymes in these pathways are the same; all of which could contribute to the observed effect. For instance, the upregulation of phenylalanine hydroxylase (PH), tyrosine hydroxylase (TH), dopadecarboxylase (DDC), and/or dopachrome conversion enzyme (DCE) could have unintended consequences for immune defense. Tyrosinase phenoloxidase (PO) may also be involved, considering that PO is actively transported to the procuticle from the hemolymph (Asano and Ashida, 2001), may play a

non-essential role in some cuticle pigmentation (Gorman and Arakane, 2010), and is generally found to be in greater abundance in darker individuals (Wilson et al., 2001; Fedorka et al., 2013b). Furthermore, natural and artificial selection for darker cuticles tends to result in increased PO abundance (Armitage and Siva-Jothy, 2005; Fedorka et al., 2013b). Future work should attempt to identify the key genes shaping this relationship.

It is important to note that other pleiotropy-independent mechanisms may underlie the association between temperature, cuticle color and immune function, thereby refuting our TDII hypothesis. For instance, temperature increases the rate of metabolic processes in insects. If increased temperature independently increases the rate of cuticle sclerotization and PO activity, then less melanin may be deposited in the cuticle (due to less time available) and less PO produced (due to a greater PO efficiency at higher temperatures). However, this seems unlikely considering that (1) positive correlations between cuticle color and immunity that are temperature independent have been well documented in other insect systems (Wilson et al., 2001), (2) it implies that natural selection does not adaptively shape cuticle color to improve thermoregulation; contrary to the evidence (Watt, 1968; Kingsolver, 1983; Majerus, 1998; Clusella Trullas et al., 2007), and (3) strong evidence for a genetic correlation between these traits exists (Armitage and Siva-Jothy, 2005; Rolff et al., 2005; Fedorka et al., 2013b). It is also important to note that other immune components are activated during a bacterial insult, such as antimicrobial peptides (AMPs), which may minimize the relative antibacterial role of melanin-based immunity (e.g. Leclerc et al., 2006). However, it is currently unknown if rearing temperature (not infection temperature) influences AMP defense, and we know of no *a priori* reason to expect such a relationship (although this relationship remains to be tested). We also note that our conclusions are limited by the use of a single pathogen. Given that host-pathogen interactions are complex, more pathogens or pathogen types should be examined to determine the scope of the thermoregulatory-dependent immune investment hypothesis.

Due to the difficulties associated with accurately measuring the body size of non-gel imbedded live individuals and desiccating dead individuals, we did not measure size in our mortality assay. Considering that cold developing individuals were larger, they may have received a smaller dose of bacteria per unit of body mass. One could therefore argue that the differences in mortality were the result of differences in size and not immune investment. Previous work in *A. socius*, however, was able to disentangle these effects and found that the degree of melanization was associated with mortality and not size (Fedorka et al., 2013b). Our study also did not disentangle the effects of temperature and photoperiod on cuticular melanization and mortality. Although photoperiod and temperature are closely related, photoperiod appears to be the most reliable cue for organisms in predicting seasonal changes in the thermal environment (Nelson and Demas, 1996; Nelson, 2004). Natural selection should therefore favor photoperiod over temperature as a cue for melanin investment; but this also remains to be tested.

In summary, we suggest that thermoregulatory strategy is tightly associated with melanin-based immune investment. Furthermore, this pattern appears to stretch across both the holometabolous and hemimetabolous insect clades, suggesting that thermoregulatory-dependent immune investment is an evolutionarily conserved phenomenon. This phenomenon should have a significant impact on the susceptibility of hosts developing in warm environments to a wide range of pathogens and could be of great importance in systems where insect hosts provide critical ecosystem services. Moreover, TDII may increase the prevalence of insect-vectored disease in warm environments due to the reduced immune investment of the vector. Future work should examine the extent to which TDII influences host mortality rates in the wild and the ability to vector pathogens in a warming climate. In addition, it would be of interest to understand how crypsis, sexual selection, aposematism, and mimicry influence thermoregulatory strategy and immunity.

MATERIALS & METHODS

Fly Stocks and Maintenance

Adult flies were collected in August of 2012 from two different Canadian localities including Massey (MO) and Sudbury (SO) Ontario. Both populations are at 40° latitude and approximately 95km apart. From these wild caught flies, 60 isofemale lines were established (30 from each population) and maintained on a cornmeal, yeast, sugar, and agar food medium for 7 generations at 25°C with a 12:12 L:D photoperiod using standing incubators (Percival, Perry, IA, USA). For each population, 6 virgin individuals (3 of each sex) from each isofemale line were randomly chosen to establish a large, outbred population cage ($30cm^3$). Prior to experimentation, each population cage was maintained at 25°C with a 12:12 L:D photoperiod for 4 generations.

Experimental Design

From each population cage, 480 virgin males and females were collected and placed into sex-specific 36ml food vials at a density of 15 individuals per vial. After four days, one male and female vial were combined into a large 200ml food bottle and allowed to mate for 48 hours. Males were then discarded and the remaining 15 females were moved to a fresh food bottle for oviposition. After 24 hours, females were removed and the oviposition bottle was randomly assigned to either a summer or fall seasonal treatment. Each experimental treatment was replicated in two different incubators. The summer treatment conditions were 28.5°C and a 14L:10D photoperiod, while the fall treatment conditions were 21.5°C with an 11L:13D photoperiod. Treatment conditions were selected to reflect average summer and fall conditions and photoperiod at their originating localities. In total, four bottles per incubator, per treatment per population were established.

Next generation virgin adults were separated by sex upon eclosion and maintained on 36ml food vials at densities of 30 flies. Flies were aged 3 days in treatment conditions and then transferred to a common environment of 25°C and a 12:12 photoperiod for 24 hours before assaying cuticular melanization and immune function. Flies were placed in a common environment to minimize the effects of transient temperature-induced differences in host physiology, metabolic rate and pathogen replication rate.

Immune Assay

To assay immune defense, a subset of flies (4 days old post eclosion) from the common environment were randomly chosen (with respect to treatment, sex and population) and infected with an approximate LD_{50} of the gram-negative bacterium *Pseudomonas aeruginosa*. To this end, flies were lightly CO_2 anesthetized and a 0.2mm diameter needle (Fine Science Tools) was dipped in bacterial solution and inserted into the mesopleuron below the wing (methods similar to Radhakrishnan and Fedorka, 2012). To create the LD_{50} concentration, bacteria were incubated in sterilized LB broth at 37°C until log phase. This solution was diluted with sterile broth to an absorbance of 0.3 at 490 nm using a microplate reader (Bio-Rad Model 680, Hercules, CA, USA). The new solution was further diluted to a concentration of $5x10^{-3}$, which corresponds to an ~LD_{50} for flies maintained at 25°C and a 12:12 L:D photoperiod. The final bacterial solution was divided into 0.5ml aliquots and stored at 4°C. Prior to use, each aliquot was removed from refrigeration, vortexed and pipetted into a shallow tube cap in which the infection needle was dipped. Bacterial solution aliquots were used for no longer than 30 minutes before being replaced with a fresh solution. Between infections, the needle was dipped in alcohol and allowed to air dry.

After infection flies were placed individually into 1.5ml tubes containing a small amount of food and capped with cotton. Survival over the next 72 hours was then recorded. By 72 hours, flies have either recovered from infection or have died, as little death occurs in days 3 and 4 post infection. Flies that did not survive the initial hour after infection were removed from analysis (n = 33), as their deaths were assumed to be due to trauma associated with needle insertion and not bacterial infection. All infections were conducted at 1pm in the afternoon under ambient laboratory conditions. In total, 1353 flies were infected by two researchers across 4 replicates per researcher. Each replicate always contained a similar number of flies from each sex, treatment and population. Only one of the two researcher performed infections blindly relative to treatment. These data were analyzed using a General Linearized Model with a binomial distribution and a logit link function. The model was constructed using treatment, sex, population, researcher and incubator nested within treatment as main effects and mortality as the binomial response. All non-significant first-order interactions were removed from the final model.

Cuticular Melanization Assay

To assay cuticular melanization, flies (4 days old post eclosion) from the common environment were randomly chosen (with respect to treatment, sex and population) and digitally photographed using the BK Plus Lab Imaging System (Visionary Digital, Los Angeles, CA, USA), which standardizes lens height, zoom, and flash to produce consistent high resolution pictures. Flies were placed in a water-based, biologically inert, colorless gel and similarly oriented. The degree of melanization was measured as the mean grayscale darkness of the image pixels using ImageJ software (http://imagej.nih.gov), with 0 being completely white and 255 being completely dark. In total, ten flies per sex, per incubator, per treatment, per population were assayed, totaling 160 flies.

Considering that most insects exhibit Bergmann's size rule or its inverse (Blanckenhorn and Demont, 2004), and that body size is expected to positively covary with cuticle darkness (Clusella Trullas et al., 2007), neglecting body size variation could lead to Type I or Type II error, respectively. To minimize this possibility, we included body size in our analysis by measuring each individual's pronotum length. To test for an effect of treatment on melanization, we used a multi-factor ANCOVA with treatment, population, sex and incubator nested within treatment as the main effects and pronotum length as the covariate. Again, all non-significant first-order interactions were removed from the final model. All analyses were conducted in JMP version 11.

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COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

Project concept and design was provided by K.M.F. and H.S., data collection was provided by H.S., I.K. and T.W., data analysis was provided by I.K. and the manuscript was written by I.K., and K.M.F.

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FIGURE LEGENDS

Fig.1. Cuticle-melanin and immune-melanin pathways. Enzymes that are shared between the pathways, cuticle-specific and immune-specific are depicted in black, dashed and white blocks, respectively. The immune-melanin pathway is generally localized to the hemocoel (solid vertical line, left side), while the cuticle-melanin pathway is localized to the epidermal cells and procuticle (dashed vertical line, right side). Asterisks depict non-enzymatic reactions. Enzyme abbreviations are as follows: phenylalanine hydroxylase (PH), dopadecarboxylase (DDC), dopachrome conversion enzyme (DCE), tryrosinase phenoloxidase (PO) and laccase phenoloxidase (Lac).

Fig.2. The influence of environmental treatment on cuticular melanization and pathogen induced mortality. Individuals who developed in summer-like environment exhibited lighter cuticle color (indicated by a lower PC1 value) and inferior resistance to the gram negative pathogen, *P. aeruginosa,* compared with those that developed in a fall-like environment. Black and white circles represent the SO and MO populations, respectively. Circles and bars represent the LS means (after controlling for sex) and associated standard error.

Fig.3. Hypothetical model for how rearing temperature and ambient temperature may influence melanin-based immune defense. As ambient temperature increases, insect metabolic rates increase, which has been shown to increase immune defense (see discussion). This implies that pathogen defense may improve with a warming climate. However, our data suggests that as rearing temperature increases (e.g. from cool to hot), insects alter their thermoregulatory strategy to create cuticles with less melanin, which in turn reduces investment in melanin-based immunity. Thus, insects reared under hot conditions (white circles) tend to exhibit light cuticles and inferior melanin-based immunity compared with insects reared under cool conditions (black circles). This observation suggests that as climates warm over the next several decades, insects may exhibit reduced defense against a wide range of pathogens. Hypothetical curves are based on $Q_{10} = 2.0$, which states that metabolic rates double with every 10ºC increase in ambient temperature; a commonly observed trend in insects (Nespolo et al., 2003).

Figure 1

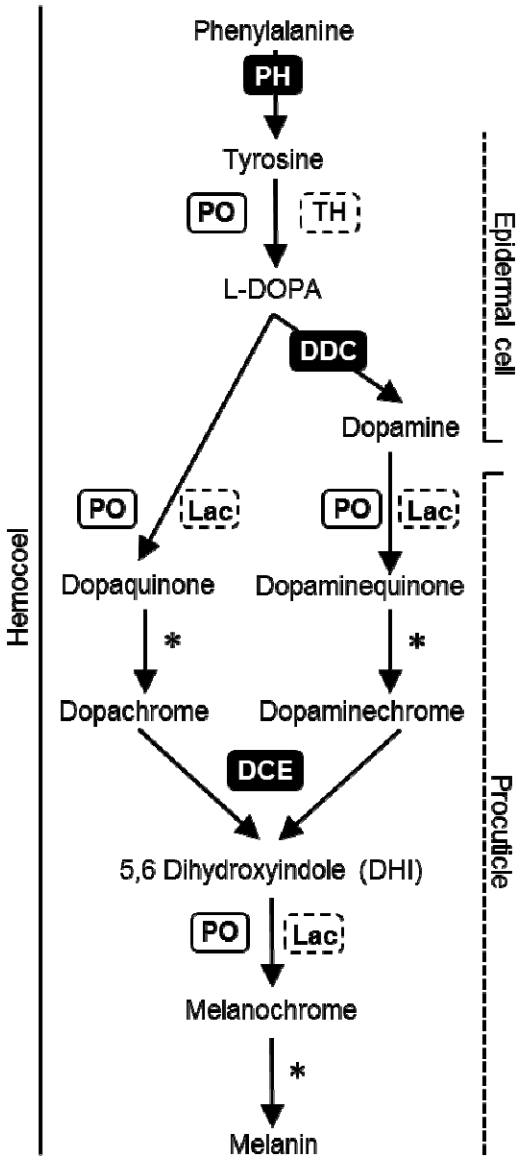

Figure 2

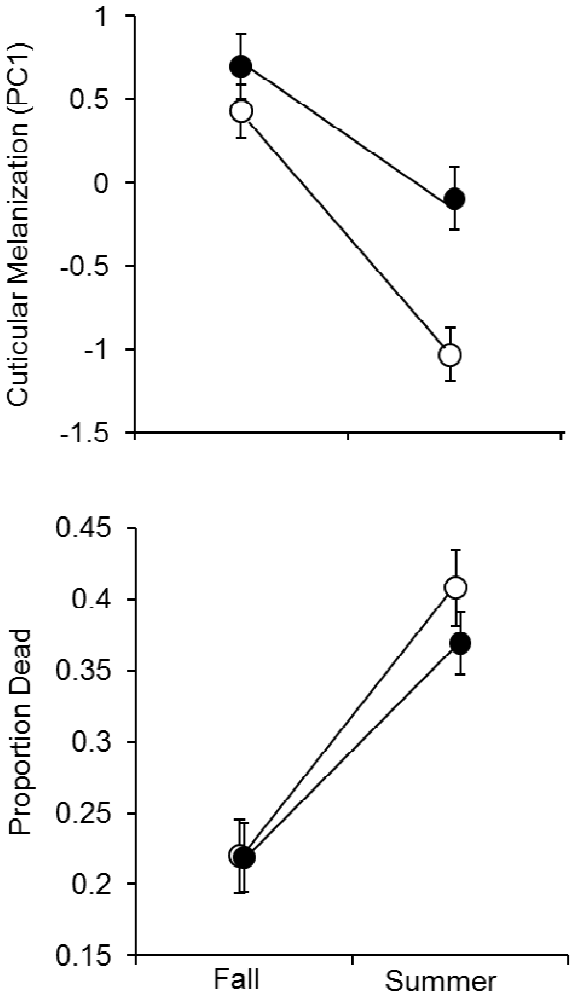

Figure 3

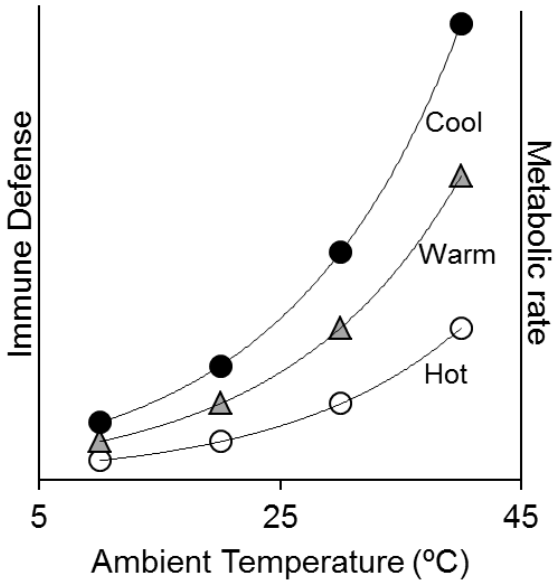

Table 1. The influence of environmental treatment on body size, cuticular melanization and pathogen-induced mortality. Table abbreviations are as follows: population = pop and treatment = treat. Bold indicates significance at the alpha = 0.05 level.

Source	d.f.	F	P
Response = Body size			
Model	5	72.4	**<0.0001**
Treat	1	56.8	**<0.0001**
Pop	1	6.24	**0.0137**
Sex	1	292	**<0.0001**
Incubator (Treat)	2	0.72	0.4885
Response = Cuticular Melanization			
Model	7	9.94	**<0.0001**
Treat	1	37.7	**<0.0001**
Pop	1	13.9	**0.0003**
Sex	1	2.08	0.1514
Pronotum	1	1.48	0.2252
Incubator (Treat)	2	1.87	0.1577
Pop X Treat	1	5.38	**0.0218**

Source	d.f.	X2	P
Response = Mortality			
Model	6	47.1	**<0.0001**
Treat	1	44.7	**<0.0001**
Pop	1	1.02	0.3106
Sex	1	1.29	0.2560
Researcher	1	0.06	0.8005
Incubator (Treat)	2	0.97	0.6153