Paling in Comparison: The Role of Sex and Temperature in Melanin-Based Immune Function

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Dedication

This thesis is dedicated to my father, Ira Ehrlich, for his enthusiastic support of my journey in higher education and to my grandparents, Bernie and Dottie Novatt, for the many opportunities they have provided me, our family, and the community.

Abstract

Sex differences in disease susceptibility have been observed across a wide range of species, yet we lack a strong understanding of how environmental context influences these patterns. In this thesis, I take a life-history approach to investigating the plasticity of immunological sex differences in insects. Insects rely on the pigment melanin for both immune function and the structure and coloration of the cuticle (i.e. integument). Although many studies have shown evidence of correlations between immunity and cuticle pigmentation, most do not take into account the many potential modes of selection acting on cuticle melanism. Given that the biochemical precursors of melanin are a common currency in thermoregulation, sexual ornamentation, and immune function in insects, we hypothesized that sex differences in melanin-based resource allocation contribute to sex differences in immunity, and that the thermal environment will moderate this influence. My thesis work focused on the interactive effects of sexual selection and thermal selection on cuticle melanism and immunity in the Pacific field cricket (*Teleogryllus oceanicus*). After rearing crickets under multiple temperatures, we found, as predicted, that females invested more in immunity, males invested more in cuticle melanism, and both immune function and cuticle melanism were reduced in individuals that developed under warmer temperatures. Our results suggest that sex-specific investment in melanin corresponds with sex differences in immunity and that thermoregulation may act as an immune constraint under high temperatures, thus regulating the extent to which males and females diverge in disease susceptibility. By quantifying melanin-based traits underlying strategies for both reproduction and survival

in insects, this study sheds light onto the selective forces shaping sex differences in immunity and insect life-history in general.

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Introduction

Sex differences in disease susceptibility have been noted in a substantial number of taxa (Butterworth et al. 1967; Zuk & McKean 1996; Moore & Wilson 2002; Nunn et al. 2009). Males are often considered the "sicker sex" because they tend to have greater disease prevalence and shorter lifespans compared to females (Zuk 2009). Sex-specific life-history strategies may be the ultimate driver of these immunological sex differences (Zuk 1990; Zuk & McKean 1996; Rolff 2002; Moore & Wilson 2002; Nunn et al. 2009). Life-history theory assumes that all organisms balance the costs of reproduction with the costs of survival (Williams 1966). This balance plays out differently for males and females because they differ in reproductive strategy: a female's reproductive success tends to be limited by the number of ova she can produce and offspring she can rear while male reproductive success is typically limited by the number of mates the male can access and inseminate (Bateman 1948; Collet et al. 2014). Thus, females are expected to maximize fitness by living longer (e.g., investing in immunity) while males are expected to prioritize investment in current reproduction at the expense of other life-history traits such as immune defense (Zuk & McKean 1996; Rolff 2002).

While this life-history hypothesis has been very helpful for our evolutionary understanding of sexual dimorphism in disease susceptibility, it remains unclear how organism-environment interactions influence this phenomenon. This is problematic given that theoretical work suggests that environmental context and ecological feedbacks can greatly alter the observed differences between male and female immunity (Restif & Amos 2010; Bacelar et al. 2011) in addition to sexual selection (Stoehr & Kokko 2006). One fruitful avenue for addressing this gap in knowledge is studying sex-specific ornaments that rely on the same resource pool as immune function. If the production and maintenance of a sexual ornament requires a limiting resource, then there will be less of that resource that can be allocated to other fitness-related functions including immunity (Sheldon & Verhulst 1996). This relationship becomes complicated, however, when accounting for the many environmental pressures acting on the ornament, thus shaping the resource pool with potential to indirectly shape immune function. For example, carotenoids, which produce yellow, orange, and red coloration, are used as a common

currency for sexual signaling and immune function in birds and other taxa, and they can be influenced by numerous environmental factors such as humidity and food availability (Olson & Owens 1998). After manipulating the amount of carotenoids in the diet of male zebra finches, Blount et al. (2003) found changes to both immune function and sexual ornamentation: carotenoid-supplemented males had more attractive sexual signals (i.e. redder bills) and a greater cell-mediated immune response. Despite such well-documented connections between sexual ornaments and immunity, there is a need to apply the implications of these sex-specific interactions with the environment towards understanding patterns of sex differences in immunity.

In insects, a great deal of research has focused on the relationship between immunity and cuticle (i.e. integument) color because both largely rely on the pigment melanin (Ashida & Brey 1995; see Appendix I for more detail). Eumelanin darkens the insect cuticle and is responsible for the diverse range of black and brown pigmentation across arthropods (Majerus 1998). Additionally, melanogenesis is important in several roles for insect immunity, including encapsulating pathogens that breach the hemocoel (encapsulation response), repairing injuries to body tissues, and some antimicrobial properties (González-Santoyo & Córdoba-Aguilar 2012; Nakhleh et al. 2017). Because these melanin-based immunological processes share biochemical pathways with cuticle melanin (Sugumaran 2002), and because melanization is costly for insects (Lee et al. 2008; Roff & Fairbairn 2013), changes to one of these traits is expected to have indirect effects on the other. Indeed, many insect species exhibit phenotypic and genetic correlations between cuticle color and melanin-based immunity; however, there is notable variation in the directions of these correlations (see Appendix II).

In this study, we focus on the potential interactive effects of sexual selection and thermal selection on cuticle melanism and insect immunity. Many insect species use melanin for sexual signals to attract mates or recognize conspecifics, and temperature is widely known to affect insect pigmentation (Majerus 1998). The theory of thermal melanism proposes that under identical thermal conditions and similar body size, ectotherms that are more heavily melanized will heat faster and maintain higher equilibrium temperatures (Clusella Trullas et al. 2007). Individuals that are lighter in

color may have an advantage at higher temperatures because they are less likely to overheat in comparison to their darker counterparts (de Jong et al. 1996; Clusella Trullas et al. 2007). Many studies have shown that insects living in warmer climates are typically lighter in color (Zeuss et al. 2014). Furthermore, these less-melanized morphs are consequently more susceptible to disease than darker morphs (Fedorka et al. 2013a,b; Kutch et al. 2014), implying that direct thermal selection on cuticle color may indirectly constrain immunity. While the exact mechanism is unknown, high temperatures during development likely cause reduced investment in cuticle melanism which, in turn, affects melanin-based immunity (Kutch et al. 2014). Because there are many shared components of the biochemical cascade between cuticle melanism and melanin-based immunity, the genes underlying these components may be responsible for this correlated response.

Given that the precursors of melanin are a common currency in thermoregulation, sexual signaling, and immune function in insects, we hypothesized that sex differences in melanin allocation contribute to sex differences in immunity, and that the thermal environment moderates this influence. More specifically, we expected that the more-melanized sex would experience resource allocation trade-offs between body pigmentation and immune function. If warmer temperatures limit the availability of biochemical resources used for melanin synthesis, then the extent to which males and females differentially invest in melanin-based traits may be amplified depending on which functions they prioritize. In warmer climates, then, both sexes would be expected to reduce investment in cuticle melanism and consequently immune function; however, the reduced investment in cuticle melanism would be less extreme in males than females because they are constrained by the need to maintain the melanin-based sexual signal.

We tested this hypothesis with the Pacific field cricket (*Teleogryllus oceanicus*). Like other crickets, male *T. oceanicus* produce an acoustic sexual signal to attract mates by rubbing together melanized structures on the cuticle of their forewings (Bennet-Clark 2003). Only male crickets sing, and these wing components are absent in female crickets. In addition to attracting females, male calling song in *T. oceanicus* also attracts a parasitoid fly (*Ormia ochracea*) in some parts of the cricket's range. After a fly finds a signaling male, she deposits larvae that burrow into the cricket, eventually killing it

(Cade 1975). Some populations of *T. oceanicus* exhibit a sex-linked wing mutation, referred to as "flatwing," that produces wings with greatly reduced song structures, thus eliminating the ability to produce song (Zuk et al. 2006).

Exhibiting both inter-sexual and intra-sexual wing dimorphism, this study system provides a unique opportunity to investigate whether sex and morph differences in immunity reflect sex and morph differences in cuticle melanism. Does maintenance of the melanized, cuticular structures used for singing in normal-wing males come at a cost to immune function, and is this cost therefore absent in both flatwing males and females? Furthermore, we were able to raise *T. oceanicus* in the lab under temperature-controlled conditions to investigate the potential for indirect effects of thermoregulation on sex differences in immune function. Specifically, we predicted (1) sex and morph differences in melanin-based traits such that females will have superior melanin-based immunity, normal-wing males will have darker cuticles, and flatwing males will fall intermediate; (2) melanin-based immunity and cuticle melanism would be reduced in individuals that developed under warmer temperatures; and (3) normal-wing males would experience this diminished immunity under increased rearing temperatures more than flatwing males and females because production of the sexual signal requires a greater investment in cuticle melanism even at the cost to immunity.

Materials and Methods

STUDY SYSTEM AND REARING CONDITIONS

All *Teleogryllus oceanicus* used in this study were from an outbred laboratory colony originating from the island of Oahu in Hawaii, USA. This laboratory stock has been supplemented with eggs from wild females annually since its establishment in 1991 and sustains at least 100 breeding adults at all times. The crickets were maintained in incubators (Caron Insect Growth Chambers model 6025) at 26°C with 75% relative humidity and 12D:12L photoperiod. They were housed in 14L plastic containers with food (Purina Rabbit Chow), egg carton for cover, and water soaked cotton pads as a source of drinking water as well as egg laying substrate. We collected egg laden cotton pads from the colony once a week for 6 weeks and put each in a 1.89 L container maintained at 26°C. We checked the egg pads daily and randomly distributed hatchlings among three temperature treatments: 26°C, 29°C, and 32°C. These temperatures all fall above the average yearly temperature for Oahu (25.5°C) but are still within the range of temperatures that occur at the site of origin (11.5°C – 34.5°C; National Weather Service Forecast Office 2017). We chose these temperatures because we expected that the range would be large enough to induce a response in cuticle color, provided that cuticle color is phenotypically plastic in *T. oceanicus*, while remaining biologically relevant.

The juvenile crickets were reared in 5.7 L plastic containers within temperature-regulated incubators corresponding to their assigned treatments. All incubators were set to 75% relative humidity and 12D:12L photoperiod with temperature held constant. Food and water were provided *ad libitum*. Each individual was transferred to a separate 118 mL plastic container with food and water within 24 hours of the adult molt but remained in the same incubator. We determined the wing morph for each male at this time (flatwing or normal-wing). Although ~50% of male *T. oceanicus* observed in Oahu are flatwing, the proportion of males that are flatwing in the laboratory is approximately 14%. Therefore, the sample size of flatwing males in this study is smaller than normal-wing males and females due to our limited access to flatwing males in the laboratory stocks.

HEMOLYMPH EXTRACTION

Between 8 and 10 days post-eclosion, we transferred each individual into a 26°C incubator for 24 hours before extracting hemolymph. This provided a common environment among all crickets to help control for direct temperature effects on metabolism or enzyme activity that could confound the immunity assays since this study focuses on developmental effects. The crickets were weighed to the nearest 0.01 gram and wiped with 70% isopropyl alcohol. We then collected 4 µL of hemolymph from each individual by piercing the pronotal membrane and immediately mixed it with 55 µL of ice-chilled 1X phosphate buffered saline (PBS; 11.9 mM phosphates, 137 mM NaCl, and 2.7 mM KCl, pH = 7.4 ± 0.1, Fisher). All samples were vortexed to disrupt the hemocytes and briefly placed in ice before being moved to a -80°C freezer for later analysis. Crickets were frozen and stored at -20°C after hemolymph removal. All subsequent data collection was performed blind to temperature treatment and sex except wing measurements since sex is apparent in wing morphology.

IMMUNITY ASSAYS

In order to gain insight into how differences in cuticle melanism may affect immunity in insects, we focused on two measurements of constitutive humoral immune function: phenoloxidase activity and lysozyme-like activity. Phenoloxidase (PO) is an activating enzyme of melanin formation in invertebrates (Söderhäll and Cerenius 1998; see Appendix I) and lysozymes break down peptidoglycans in the cell walls of gram positive bacteria in the hemolymph (Schneider 1985). Although we were mainly interested in melanin-based immunity for this study, we included lysozyme-like activity to examine how temperature and sex might affect different branches of the immune system and also because, in *T. oceanicus*, PO activity and lysozyme-like activity are negatively related (Bailey and Zuk 2008). In addition to these two immune parameters, we also measured total protein of the hemolymph. Circulating protein concentrations may not only reflect an individual's condition, but also can predict resistance to a variety of bacterial pathogens in Gryllids (Adamo 2004).

To quantify total PO activity of the hemolymph, we adapted protocols from Adamo (2004) and Bailey and Zuk (2008). We added 5 µL of the hemolymph-PBS solution for each sample to 7 µL of a 1.3 mg mL^{-1} solution of α-chymotrypsin (Sigma) and incubated at room temperature for 20 minutes. Phenoloxidase is stored in the hemolymph as an inactive zymogen (prophenoloxidase) and is activated by serine proteases (Söderhäll and Cerenius 1998). The serine proteases themselves are activated by microbial cell walls, but chymotrypsin is commonly used in the laboratory to activate prophenoloxidase in the absence of infection (González-Santoyo & Córdoba-Aguilar 2012). After the incubation period, we added 90 µL of 15 mM L-3,4-dihydroxyphenylalanine (L-DOPA; Acros Organics) to the hemolymph mixture in a 96-well microplate. Phenoloxidase converts L-DOPA to dopachrome (González-Santoyo & Córdoba-Aguilar 2012) which causes the sample to become darker as the reaction proceeds. We measured this change in color at 490 nm for 120 minutes at room temperature using a Biotek EL808 microplate reader. We report PO activity as the change in absorbance over the linear phase of the reaction. Larger changes in absorbance indicate greater PO activity.

To measure lysozyme-like activity in the hemolymph, we used a turbidimetric assay described in Bailey and Zuk (2008). We transferred 10 µL of the hemolymph-PBS solution from each sample to a microplate well containing 90 µL of a 0.35 mg mL^{-1} suspension of *Micrococcus luteus* (Sigma) and took absorbance readings at 490 nm for 120 minutes. The rate of cell wall hydrolysis was inferred by the decreasing absorbance readings as the reaction proceeded and the solution became more transparent. To facilitate the interpretation of these data, we report the absolute value of the change in absorbance over the linear phase of the reaction such that higher values indicate greater lysozyme-like activity.

Total hemolymph protein was measured with a Bradford assay (Bradford 1976). We transferred 4 µL of the hemolymph-PBS solution for each sample to a microplate well and mixed it with 70 µL of Bradford reagent (Sigma). After a 10 minute incubation period at room temperature, we read the absorbance at 630 nm and compared the values

to a standard curve using bovine albumin (Sigma) run simultaneously. All standards and samples were run in duplicate for this assay.

MEASURING CUTICLE MELANISM

We measured cuticle darkness from the same individuals used for the immunity assays. After crickets were removed from the freezer and thawed, we photographed the dorsal surface of the femur of the left hindleg and the left forewing using a digital camera mounted on a stereomicroscope at 8X magnification (SPOT 5.1 Advanced Imaging Software). We chose to measure the hindleg in addition to the wing because previous work in crickets has shown that leg coloration responds to changes in the thermal environment (Fedorka et al. 2013a,b). The left side was arbitrarily chosen for consistency. We measured the mean greyscale darkness of the pixels in ImageJ software (National Institutes of Health, http://imagej.nih.gov) after converting the photos to 8 bit. To facilitate interpretation, we transformed the measured darkness values so that they ranged from 0 (completely white) to 255 (completely black). We also measured the mean greyscale darkness of three control areas in the background of each image in order to account for slight variation in lighting between photographs. These background darkness values were averaged for each photo and subtracted from the overall background average. This difference was then subtracted from the original measurement of the leg or wing for each photo. For example:

Wing cuticle darkness = mean darkness of wing – (mean background darkness of the photo – mean background darkness across all wing photos)

STATISTICAL ANALYSES

All analyses were conducted in JMP version 12.0.1 (SAS Institute Inc., Cary, NC, USA). We ran two-way ANOVAs to quantify the effects of sex and rearing temperature on each response variable with mass included as a covariate. We checked the residuals for assumptions of normality. Due to lack of normality, lysozyme-like activity, PO activity, and leg cuticle melanism were Box Cox transformed and total protein concentration was squared. Individual groups were compared *post-hoc* using Tukey-

Kramer tests. We also tested for potential phenotypic trade-offs between immunity and cuticle melanism using Pearson moment product correlations. Measurements for some individuals needed to be discarded because of human error or missing appendages (in the case of quantifying cuticle darkness), so total sample sizes are not equal across analyses. Throughout, mean values are reported 1 ± standard error.

Results

EFFECT OF SEX AND REARING TEMPERATURE ON IMMUNITY

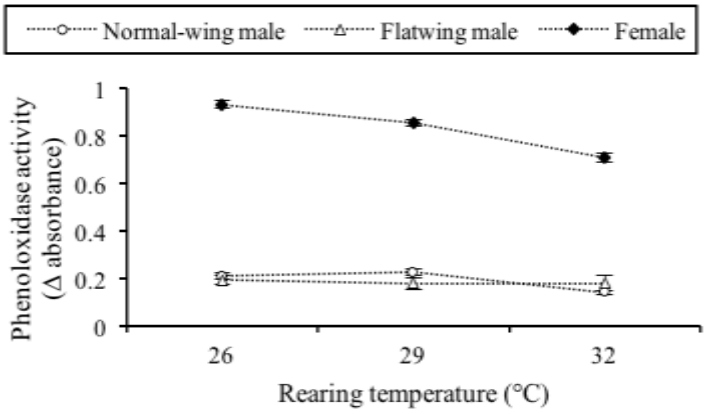
In Table 1a-c, we summarize the findings from the hemolymph analysis. Females had greater PO activity (Fig. 1a, Table 1a) and a higher protein concentration (Fig. 1c, Table 1c) than males, but the sexes did not differ in lysozyme-like activity (Fig. 1b, Table 1b). Male wing morph did not significantly affect any of the immunity measurements (PO activity: $p=0.9599$; lysozyme-like activity: $p=0.9113$; protein concentration: $p=0.0808$).

All three hemolymph measurements declined with warmer rearing environments (Table 1a-c). Overall, PO activity was lower at 32°C than at 26°C ($p<0.0001$) and 29°C ($p=0.0001$), but flatwing male PO activity did not significantly respond to rearing temperature ('Sex/Morph x Temperature' interaction, Table 1a, Fig. 1a). Futhermore, females experienced a greater decrease in PO activity from 26°C to 29°C compared to normal-wing and flatwing males. Lysozyme-like activity was also lower in individuals raised in warmer environments (26°C vs. 29°C: $p=0.0119$; 26°C vs. 32°C: $p<0.0001$; 29°C vs. 32°C: $p=0.1291$; Fig. 1b). In contrast to PO activity, however, normal-wing males displayed a greater decline in lysozyme-like activity under warmer rearing conditions than both females and flatwing males ('Sex/Morph x Temperature' interaction, Table 1b, Fig. 1b). Protein concentration (μg protein mL^{-1}) was lower for individuals that developed in 32°C than at 26°C and 29°C ($p<0.0001$ for both pairwise comparisons; Fig. 1c). There were no significant sex or morph differences in protein concentration at 26°C (females vs. normal-wing males: $p=0.6528$; females vs. flatwing males: $p=0.0959$; normal-wing males vs. flatwing males: $p=0.6251$) but there were sex differences at 29°C (females vs. normal-wing males: $p=0.0016$; females vs. flatwing males: $p=0.0043$) and 32°C (females vs. normal-wing males: $p<0.0001$; females vs. flatwing males: $p=0.0204$). This was likely due to males expressing slightly more pronounced drops in protein concentration under higher temperatures than females (Fig. 1c) even though there was a marginally nonsignificant 'Sex/Morph x Temperature' interaction for protein concentration (Table 1c).

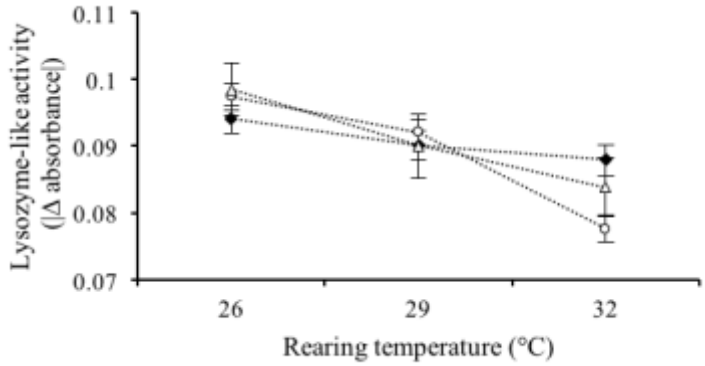
Mass was only implicated in lysozyme-like activity, such that heavier individuals tended to have greater activity ($r^2 = 0.023$, $p<0.0001$, Table 1b). However, this relationship was only apparent in males.

Figure 1: Influence of temperature during development on adult (a) PO activity, (b) lysozyme-like activity, and (c) protein concentration in the hemolymph of *Teleogryllus oceanicus*. Sample sizes per temperature group range from 104-109 for females, 99-119 for normal-wing males, and 13-22 for flatwing males. Phenoloxidase activity and lysozyme-like activity were measured spectrophotometrically via colorimetric assays such that greater change (Δ) in absorbance corresponds with greater immune enzyme activity.

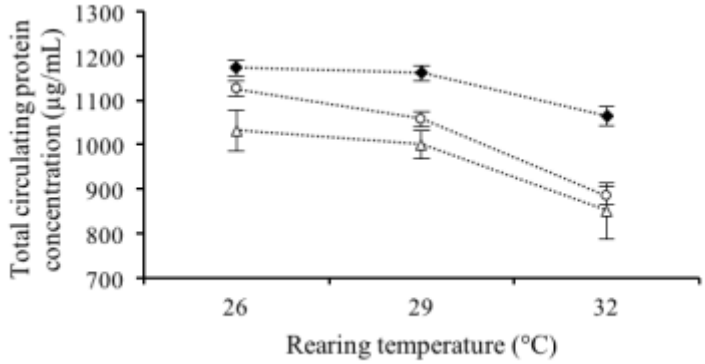
(a)



(b)



(c)



EFFECT OF SEX AND REARING TEMPERATURE ON CUTICLE MELANISM

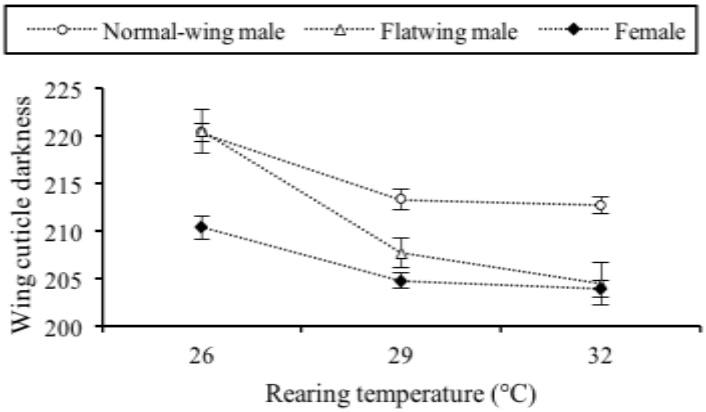
Table 1d,e summarizes the results from the cuticle coloration measurements. Females had lighter cuticles than males for the wing (females vs. normal-wing males: $p<0.0001$; females vs. flatwing males: $p=0.0047$; Fig. 2a) and the hindleg (females vs. normal-wing males: $p<0.0001$; females vs. flatwing males: $p<0.0001$; Fig. 2b). Furthermore, normal-wing males had darker wings than flatwing males ($p=0.0053$) but the morphs were not significantly different in hindleg color ($p=0.7569$).

Crickets in the 26°C treatment had darker wings than crickets in 29°C and 32°C ($p<0.0001$ for both pairwise comparisons; Fig. 2a). Although there was not a significant 'Sex x Temperature' interaction for wing cuticle color (Table 1d), flatwing males displayed a greater difference in wing cuticle color between the 26°C and 29°C treatments on average than females and normal-wing males (Fig. 2a).

Crickets raised at 29°C had lighter hindlegs than those raised at 26°C ($p<0.0014$) and 32°C ($p<0.0001$; Fig. 2b) and larger individuals had lighter colored legs ($r^2 = -0.048$, $p<0.0001$, Table 1e). We would like to highlight that, although statistically significant, the differences in cuticle darkness across rearing environments are small for the wing (26°C: 215.82 ± 0.79, 29°C: 208.84 ± 0.68, 32°C: 208.37 ± 0.67) and especially the leg (26°C: 211.02 ± 0.81, 29°C: 209.16 ± 0.72, 32°C: 212.72 ± 0.73).

Figure 2: Effects of rearing temperature on cuticle melanism of the (a) forewing and (b) hindleg femur of female, normal-wing male, and flatwing male *Teleogryllus oceanicus*. Sample sizes per temperature group range from 93-108 for females, 94-124 for normal-wing males, and 13-22 for flatwing males.

(a)



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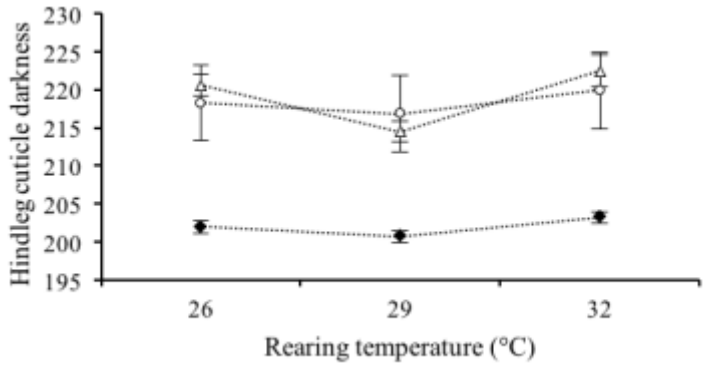


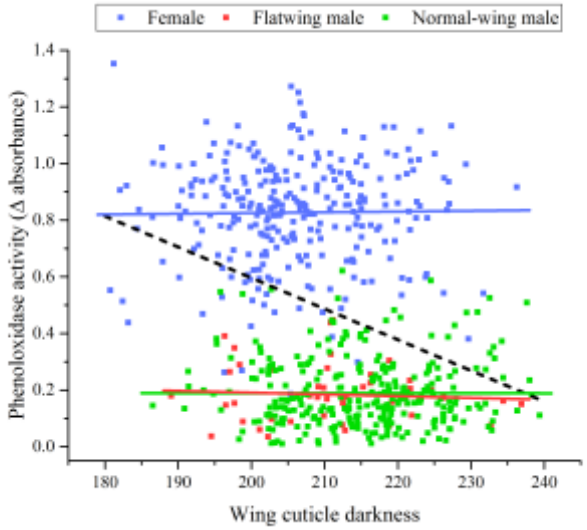
RELATIONSHIPS AMONG IMMUNE PARAMETERS AND CUTICLE MELANISM

Because trade-offs between immune system effectors are commonly reported if there are antagonistic genetic or phenotypic correlations, we investigated the relationship of PO activity, lysozyme-like activity, and total protein concentration to one another. We found that lysozyme-like activity increased with PO activity (r^2=0.024, $F_{1,679}$ = 16.611, p<0.0001). However, within temperature treatments there was only a positive correlation between lysozyme-like activity and PO activity at 32°C (r^2=0.08, $F_{1,218}$ = 18.93, p<0.0001); there was no correlation between PO activity and lysozyme-like activity within the 26°C ($F_{1,218}$ = 0.27, p=0.6067) and 29°C treatments ($F_{1,227}$ = 0.35, p=0.5542). Total protein concentration in the hemolymph increased with PO activity (r^2=0.182, $F_{1,677}$ = 150.72, p<0.0001) and with lysozyme-like activity (r^2=0.054, $F_{1,687}$ = 39.06, p<0.0001). Furthermore, we found that leg cuticle darkness increased with wing darkness (r^2 = 0.29, $F_{1,672}$ = 274.63, p<0.0001).

Although across all data there was a negative relationship between cuticle melanism and PO activity (wing: r^2= -0.115, $F_{1,627}$ = 81.79, p<0.0001; leg: r^2= -0.456, $F_{1,624}$ = 522.74, p<0.0001), this trade-off largely disappeared once sex was accounted for (Fig. 3). Correlation analyses did not indicate a relationship between PO activity and wing cuticle darkness within each sex and morph (females: $F_{1,286}$ = 0.07, p=0.7899, flatwing males: $F_{1,46}$ = 0.03, p=0.8697, normal-wing males: $F_{1,291}$ = 0.01, p=0.9213; Fig. 3a). Leg cuticle darkness (Fig. 3b) also did not correlate with PO activity within males (flatwing: $F_{1,45}$ = 1.09, p=0.3023, normal-wing: $F_{1,288}$ = 0.29, p=0.5882). However, females showed evidence of a trade-off between hindleg cuticle melanism and PO activity (r^2= -0.028, $F_{1,287}$ = 8.16, p=0.0046).

Figure 3: Relationship between phenoloxidase activity and cuticle darkness of the (a) forewing and (b) hindleg of *Teleogryllus oceanicus*. The black, dashed least squares regression lines show the relationship across all data while the solid regression lines correspond to the relationship within females (n_{wing}=288, n_{leg}=289), flatwing males (n_{wing}=48, n_{leg}=47), and normal-wing males (n_{wing}=289, n_{leg}=290).

(a)



(b)

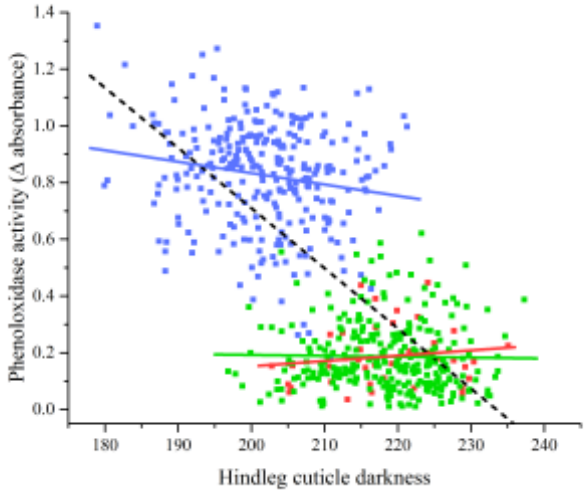


Table 1: Analysis of covariance results for the effect of sex, male wing morph, rearing temperature, and mass (covariate) on immunity, protein concentration, and cuticle melanism in *Teleogryllus oceanicus* adults. P<0.05 in bold.

Response	d.f.	F	P
(a) Phenoloxidase activity (r^2 = 0.826)			
Sex/Morph	2	1340.01	< **0.0001**
Temperature	2	14.02	< **0.0001**
Temperature x Sex/Morph	4	3.51	**0.0075**
Mass	1	0.01	0.9340
Error	670		
(b) Lysozyme-like activity (r^2 = 0.096)			
Sex/Morph	2	0.21	0.8128
Temperature	2	10.72	< **0.0001**
Temperature x Sex/Morph	4	3.04	**0.0168**
Mass	1	14.13	**0.0002**
Error	680		
(c) Total circulating protein (r^2 = 0.211)			
Sex/Morph	2	29.63	< **0.0001**
Temperature	2	21.48	< **0.0001**
Temperature x Sex/Morph	4	2.21	0.0663
Mass	1	0.38	0.5385
Error	693		
(d) Wing cuticle melanism (r^2 = 0.247)			
Sex/Morph	2	67.56	< **0.0001**
Temperature	2	35.1	< **0.0001**
Temperature x Sex/Morph	4	1.9	0.1069
Mass	1	0.44	0.5080
Error	676		
(e) Leg cuticle melanism (r^2 = 0.542)			
Sex/Morph	2	355.38	< **0.0001**
Temperature	2	13.04	< **0.0001**
Temperature x Sex/Morph	4	1.41	0.2306
Mass	1	7.16	**0.0076**
Error	674		

Discussion

Sex differences in disease susceptibility are ubiquitous in nature, yet we lack a universal framework for predicting them because we do not know the role that organism-environment interactions play. In this study, we investigated whether male melanin-based ornamentation corresponded with lower immune function compared to females, and whether temperature shaped these sex differences in immunity given it is a known selective pressure on cuticle coloration. Our results suggest that the immunological sex differences in *Teleogryllus oceanicus* may be partially driven by differential resource allocation of melanin precursors between cuticle darkness and immunity. Furthermore, we found that the temperature during development moderated the magnitude of sex differences in immunity.

Although most evidence of sex differences in disease susceptibility has derived from vertebrates, insects are a prime system for studying this phenomenon. Insects lack testosterone and estrogen which presents the opportunity to study sexual dimorphism in disease susceptibility outside of the confounding effects of these sex steroid hormones (Grossman 1985). Our results using the Pacific field cricket corroborate other studies that suggest sex-specific life-history strategies are sufficient predictors of divergence in male and female immune function (Rolff et al. 2002). We found that melanin-based immunity (PO activity) was greater in females than males, agreeing with previous work with *T. oceanicus* (McNamara et al. 2014) and several studies using other cricket species (Adamo 2001; Fedorka et al. 2004; Bailey 2011; but see Fedorka et al. 2013b). More generally, a meta-analysis of insect literature found that females tend to have greater PO activity (Nunn et al. 2009). Total protein concentration in the hemolymph was also greater in females than males in our study. It is unknown whether protein concentration predicts resistance to bacterial pathogens in this species, but increased resistance has been demonstrated in another cricket species, *Gryllus texensis* (Adamo 2004). If this is also the case in *T. oceanicus*, then females are more resistant to disease than males in addition to having higher PO activity. We did not find sex differences in lysozyme-like activity, again corroborating results from a previous study with *T. oceanicus* (McNamara et al. 2014). Because lysozymes are not directly implicated in the biochemical pathways for

melanin production, the similarity of male and female lysozyme-like activity in comparison to PO activity lends support to the hypothesis that sex differences in investment of melanin-based resources contribute to sex-specific immune function.

While females tended to have greater immune function compared to males in this study, the magnitude of these differences changed under warmer rearing environments. Males experienced greater decreases in lysozyme-like activity and protein concentration with increasing rearing temperatures, causing a greater divergence between males and females (Fig. 1b,c). This is expected if males are more likely to invest in reproductive efforts than immune function. However, females experienced a greater decrease in PO activity than males under higher rearing temperatures, and sex differences in PO activity therefore became less pronounced (Fig. 1a). Female PO activity may have been more affected by higher rearing temperatures than that of males because females use PO for egg production (González-Santoyo & Córdoba-Aguilar 2012), creating a greater need to divert melanin precursors to reproduction under stressful conditions.

Melanin-based immunity (PO activity) was lower in individuals that developed in warmer conditions, possibly because of a reduction in melanin synthesis as proposed in previous work (Fedorka et al. 2013 a,b). However, total circulating protein concentration and lysozyme-like activity also decreased with rising temperatures. It is possible that the increased temperatures acted as a stressor, thus downregulating immune function in general. This stress could be a result of faster development times under higher temperatures: the crickets reared at 32°C and 29°C had much shorter development times compared to the crickets that were reared at 26°C (see Appendix III, Fig. 4). It remains unclear whether temperature effects on development time had a greater influence on immune function than temperature effects on cuticle color because our experimental design does not allow us to disentangle these processes. Regardless of the exact mechanism, the plasticity of these immune parameters across thermal conditions may have implications for disease dynamics as temperature affects growth, behavior, and survival of hosts *and* pathogens (Linder et al. 2008; Adamo & Lovett 2011).

Our findings show limited changes in cuticle color across rearing temperatures. Wing cuticle color was lighter for individuals that developed under warmer conditions,

but the effect may not be due to thermoregulation given the small, non-linear response to temperature in leg cuticle darkness. However, considering that the wings cover much of the dorsal surface of the crickets, they might be more important for solar absorption in this species and are accordingly more responsive to temperature. Most research into thermal melanism has focused on comparing cuticle coloration either among geographically distinct populations and species (e.g., Davis et al. 2005; Fedorka et al. 2013b; Zeuss et al. 2014) or within seasonally polyphenic species (e.g., Stoehr & Wojan 2016), and therefore little is known about plasticity in cuticle coloration within a continuously breeding population. *Teleogryllus oceanicus* is a subtropical species with aseasonal reproductive activity (approximately four generations per year; Otte & Alexander 1983). There is not a large range in temperature in Oahu, the laboratory population's site of origin, and therefore other environmental factors might play a more prominent role in shaping cuticle coloration. Humidity has been shown to influence cuticle melanism in some tropical species because individuals with darker cuticles are believed to be more resistant to dessication (Parkash et al. 2009). In *Bicyclus* butterflies in Africa, patterns in wing melanization correspond with change in rainfall and humidity rather than temperature because precipitation is a more reliable cue for change in season than temperature (Roskam & Brakefield 1999). Therefore, humidity may be a major driver of cuticle melanism in the tropics, but further evidence is necessary. Future studies should consider manipulating humidity when working with tropical species in regards to immunological variation.

As expected, *T. oceanicus* males had darker cuticles than females. While we had predicted that males would have darker wings to support song production, it is not immediately clear why males have darker hindlegs than females. The positive correlation between leg color and wing color suggests that allocation to cuticle melanization may be an all-or-nothing response such that males invest more in cuticle melanin overall. Alternatively, there may be sexual selection on male cuticle darkness if females prefer darker males, but this has yet to be explored in this species. It would be beneficial to examine specifically how melanization of the wings influences calling song and female

attraction to males. This would help us understand whether male wing melanization relates to male quality and female choice.

We had predicted that flatwing males would fall intermediate in cuticle melanism and therefore also in immune function. However, we only found statistical differences between flatwing males and normal-wing males in wing cuticle color. The immunological similarity between the two male morphs refutes the idea that investment in cuticle melanism drives investment in immunity. Prior work with this system found that flatwing males have lower lysozyme-like activity (Bailey et al. 2011) and flatwing males and normal-wing males have differential immune gene expression (Pascoal et al. 2016). It is possible that we lacked the power to detect such morph differences in immunity in this study because of the small sample sizes for flatwing males. We did, however, find that females and flatwing males became more similar in wing color over changing temperatures (Fig. 2). If increasing temperature constrains melanin synthesis, then flatwing males may be more likely than normal-wing males to divert resources from wing melanism elsewhere because their mating success does not rely on the maintenance of the melanized calling structures. This signifies that reproductive strategy may act as a constraint on the plasticity to lighten cuticle color when it is assumed to be advantageous to do so.

Although we found a positive association between cuticle darkness and melanin-based immunity across temperature treatments (i.e. crickets at warmer rearing temperatures had lighter wings and lower PO activity), we found a negative association between the sexes (i.e. males have darker cuticles and lower PO activity than females), following the pattern we would expect with sex-specific investment in melanin. When we examined this relationship within sexes, however, there was inconsistent evidence of a trade-off (Fig. 3). The overall lack of a correlation between cuticle color and PO activity suggests that there is not a male-specific trade-off between these two traits as had been predicted. More generally, this finding also suggests that cuticle color does not predict melanin-based immune function within sexes in *T. oceanicus*. Previous work using another field cricket, *Gryllus campestris*, showed that males facing an immune challenge during the nymphal stage had less-melanized wings as adults, suggesting that the

maintenance of these sexually selected structures comes at a cost to immunity (Jacot et al. 2005). We may not have detected a similar trade-off if there was greater variation in resource acquisition than resource allocation for individuals in our study (van Noordwijk & de Jong 1986). The crickets had unlimited access to food during development and therefore the expected trade-off between PO activity and cuticle color in the adults may have been masked. For instance, Lee et al. (2008) manipulated the quality of protein that *Spodoptera littoralis* larvae were fed and only found evidence of trade-offs between melanization and various other life-history traits in the caterpillars after accounting for diet; there was no evidence of a trade-off within the diet treatments.

It is important to note that encapsulation response is a common metric of immune function in insects and also relies on PO activity and melanization (Gillespie et al. 1997). While we did not quantify encapsulation response for this study, Bailey and Zuk (2008) found encapsulation response and PO activity to be negatively correlated in *T. oceanicus*. Furthermore, male *T. oceanicus* have a stronger encapsulation response than females (Zuk et al. 2004). Therefore, our understanding of how cuticle melanism relates to melanin-based immunity and sex will depend on which immune parameters are measured in a given study because there is potential for negative correlations between immune traits. This is true for non-melanin based immune traits as well. For instance, mechanisms of infection tolerance like antioxidant levels may be correlated with PO-mediated immune defense (Stahlschmidt et al. 2015). It is important to document these inter-relationships between immune effector systems and especially to record multiple parameters within a given study to allow for a more accurate, holistic perspective (Adamo 2004; see discussion in Appendix II).

By quantifying melanin-based traits underlying strategies for both reproduction and survival in insects, this study sheds light onto the selective forces shaping sex differences in immunity and insect life-history in general. We found sex differences in both cuticle melanism and melanin-based immunity in *Teleogryllus oceanicus* that support the hypothesis that females invest more in immune activity while males invest more in sexual ornamentation. We also found that rearing temperature constrains immune function and moderates the magnitude of sex differences in immunity, but it is unclear

whether this is attributable to temperature-dependent cuticle melanism. Our results highlight the importance of environmental context when evaluating immune function and the need to further investigate the role of melanin-based resource allocation in the maintenance of immunological sex differences in insects. Temperature is one of the most important factors for the biology of insects, and it is important to understand how thermal selection directly and indirectly influences male and female vitality. This is especially relevant in the face of global warming and its many potential effects on pigmentation and immunity (Roulin et al. 2014). Additional study of the relationship between cuticle color, immune function, disease resistance, and the abiotic environment is much needed in tropical species which likely rely on different environmental cues for life-history decisions and may be the most impacted by climate change (Deutsch et al. 2008).

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Appendix I

Melanins are pigment-forming biopolymers found across a diverse range of organisms (Majerus 1998). Eumelanins are responsible for much of the brown and black pigmentation we see in nature, and they are the most common type of melanin found in insects (Sugumaran 2002). The enzyme phenoloxidase (PO) initiates melanin synthesis in insects and other invertebrates (Söderhäll and Cerenius 1998).

Insects utilize melanin synthesis for a variety of functions. One of its main roles is for strengthening the cuticle (Sugumaran 2002). The cuticle is a natural barrier composed of carbohydrates and protein that protects vulnerable tissues from the external environment (Andersen 2012). The strengthening process, known as sclerotization, is typically accompanied by deposition of melanin in the cuticle, thus providing pigment (Sugumaran 2002). Cuticular sclerotization is mediated by hormones as the insect develops (Andersen 2012). Phenoloxidase initiates sclerotization by oxidizing the catecholamine sclerotizing precursors to quinones which, in turn, form sclerotized products that harden and tan the cuticle (Sugumaran 2002).

Insects and other invertebrates also utilize melanin and its biochemical precursors for immune defense. Melanin and some of the intermediates (reactive quinones) released during melanin formation are cytotoxic and can thus help kill off pathogens (Nakhleh et al. 2017). When a parasite or pathogen breaches the hemocoel, it induces a cellular immune response in the insect host whereby hemocytes form a melanic capsule around the intruder, effectively sequestering it, leading to anoxia and starvation (Gillespie et al. 1997). Furthermore, melanin synthesis is also important for wound healing. When wounds form, there is often deposition of melanin at the wound sites to act as a barrier to keep hemolymph from exiting the body cavity and pathogens from entering (Nakhleh et al. 2017).

Unintended activation of PO in the hemolymph can be deleterious to insects because it leaves quinonoid toxic compounds circulating throughout the body, causing undue oxidative damage (Söderhäll and Cerenius 1998). To avoid this unnecessary damage, PO is stored in the hemolymph as an inactive zymogen precursor,

prophenoloxidase (proPO), and is only activated in response to injury or infection (Söderhäll and Cerenius 1998). Prophenoloxidase is typically synthesized and stored in circulating hemocytes, released via cell lysis when necessary, and activated by serine proteases (González-Santoyo & Córdoba-Aguilar 2012). The PO cascade is triggered by recognition of pathogen associated molecular patterns (e.g., β-1,3 glucans, peptidoglycans, lipopolysaccharides, etc.) on the membrane of hemocytes or epithelial cells via pattern recognition proteins (Gillepsie et al. 1997). This non-self recognition causes the activation of cellular and humoral immune responses.

The PO cascade begins by the hydroxylation of phenylalanine to form tyrosine (Nakhleh et al. 2017). Tyrosinase-like POs then catalyze the hydroxylation of tyrosine to dopa (dihydroxyphenylalanine) and the oxidation of dopa to dopaquinone (or to dopaminequinone if dopa is decarboxylated). Dopaquinone is non-enzymatically converted to dopachrome which is then converted to 5,6-dihydroxyindole (DHI). Eventual oxidation of DHI and polymerization of quinones generates eumelanin (Nakhleh et al. 2017). This process describes the PO cascade utilized in insect immunity, but it is important to note that the pathway is very similar for cuticle melanization: they both rely on phenylalnine and tyrosine, produce the same substrate intermediates, and share some key enzymes (Anderson 2012).

Prophenoloxidase is present in both the hemolymph and the cuticle, and therefore provides a mechanistic link between humoral immune function and cuticle pigmentation (Ashida & Brey 1995). Maintaining the PO system is costly because phenylalainine, the starting material for melanin synthesis, can only be obtained from the diet and because melanin requires a substantial amount of nitrogen which can also be a limiting resource (Stoehr 2006; González-Santoyo & Córdoba-Aguilar 2012).

Appendix II

Many studies have suggested that immunity and cuticle melanism are phenotypically and genetically linked (e.g., Wilson et al. 2001; Armitage & Siva-Jothy 2005; Bailey 2011; Busso et al. 2017). However, the implications of these links remain unclear because the studies show mixed results: some report positive correlations, others report negative ones, and still many fail to find a statistically significant relationship between cuticle melanism and immune function (Table 2). This lack of consistency brings into question how broadly the results of such studies can and should be interpreted. Should cuticle darkness be used as a predictor for immune function when there are such inconsistent findings about what a dark or light cuticle indicates? How can we explain the discrepancies?

We argue that part of this ambiguity stems from the lack of strong, clear predictions about the relationship between cuticle melanism and immunity. For example, one might draw from life-history theory and predict that cuticle melanism and melanin-based immunity will be negatively related (traded-off) as competing functions that utilize the same biochemical precursors. However, one might also predict that individuals that have access to more melanin-based resources will display both greater cuticle melanism and greater immune function compared to those that have a more limited supply. Both predictions logically follow from the hypothesis that the traits are linked, and both would provide support to the hypothesis if found to be true, yet they are in opposition to one another. Therefore, these predictions do not inform our knowledge about the relationship between cuticle melanism and immunity.

A major point of confusion is that there is little distinction made between predictions focusing on dynamics *within* individuals and those *among* individuals. One might expect a negative association between cuticle melanism and immunity within an individual given that each organism has a finite amount of resources to draw upon and allocate to different functions (Sheldon & Verhulst 1996). However, this prediction makes less sense when the point of comparison is between, rather than within, individuals. One might instead predict a positive association between cuticle melanism

and immunity among individuals, populations, or species such as when comparing different experimental treatments or discrete color morphs. Ultimately, this prediction stems from the theory that there is heterogeneity in resource acquisition across individuals, and those that acquire more resources have more to allocate to all fitness-related traits compared to those that acquire less, even if there is a trade-off at an individual level (van Noordwijk & de Jong 1986; Reznick et al. 2000). In other words, individuals that acquire more melanin-based resources or are in better condition can pay the costs of immunity and still invest in a darker cuticle (or vice-versa).

Accounting for the differences in predictions within individuals versus between individuals is a critical step towards understanding what to expect from the link between immune function and cuticle pigmentation, and for life-history trade-offs in general. For instance, even studies that share a common focus on the relationship between a melanic sexual ornament and immune function within males differ in the direction of their predictions (e.g., negative: Siva-Jothy 2000; positive: Rantala et al. 2000). This is not surprising given that the field of parasite-mediated sexual selection faces similar issues about what relationship to predict between disease susceptibility and sexual signal quality (Jacobs & Zuk 2012). In addition to distinguishing between predictions focusing on dynamics within individuals and those among individuals, future studies should also account for differences in individual quality or variation in resource acquisition when possible. This will help to gain insight into the mechanism behind a positive correlation between cuticle melanism and immunity.

Another potential source of the mixed results found in Table 2 is that many studies only measure one aspect of the immune system. If the single measured immune component is not the most relevant to defend against the pathogen(s) of interest, or if a population has evolved to focus investment in a different aspect of immunity, then the single assay may not express the expected relationship and may provide results that conflict with studies conducting a different immune assay (Adamo 2004). Even if a study does investigate multiple facets of immune defense, the measured immune components might not all relate to cuticle color in the same way, especially across species. It is common for immune effector systems to be negatively correlated with one another,

further complicating the expected relationships for cuticle melanism and immunity at large.

We propose that the work done in this thesis (summarized in the final row of Table 2) addresses several of the issues mentioned above and provides new perspective to the relationship between cuticle melanism and immunity. By investigating predictions and reporting findings both within and among groups and by measuring multiple immune traits, our work demonstrates that opposing relationships can emerge even within a single study. More work is necessary at varying scales of analysis (i.e. within and among individuals) and under different environmental conditions in order to determine whether cuticle pigmentation can be generalized as a positive or negative indicator of constitutive and realized immune function.

Table 2: Literature review of reported relationships between cuticle pigmentation and immune function (humoral phenoloxidase (PO) activity, encapsulation response, hemocyte concentration in the hemolymph, and antibacterial activity) in insects. The findings have been sorted by taxonomic order of the study species. All studies used adults unless otherwise indicated. "Positive" = darker individuals or groups had greater measures of the immune trait; "Negative" = darker individuals or groups had lower immune measures (i.e. a trade-off between cuticle darkness and immunity); "NS" = no significant relation between cuticle melanism and the immune trait at α=0.05; "-" = trait was not measured in a particular study or the necessary data were not available in the publication.

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Coleoptera						
Tenebrio molitor	Between groups raised in solitary vs. gregarious conditions	NS	-	-	-	Barnes & Siva-Jothy 2000
Tenebrio molitor	Between black vs. tan selection lines	Pre-immune challenge: Positive Post-immune challenge: NS	-	Positive	NS	Armitage & Siva-Jothy 2005
Tenebrio molitor	Within males vs. females	Females: Negative Males: NS	-	Genetic: Negative Phenotypic (females): Negative Phenotypic (males): NS	-	Rolff et al. 2005

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Coleoptera (continued)						
Tenebrio molitor	Between black vs. tan phenotypes across three temperatures	-	Low temp: Positive Medium temp: NS High temp: NS	-	-	Prokkola et al. 2013
Tenebrio molitor	Between three discrete color phenotypes	-	First immune challenge: NS Second immune challenge: Positive	-	-	Krams et al. 2016
Tenebrio molitor	Between black vs. tan selection lines	NS	-	NS	-	Evison et al. 2017
Diptera						
Sepsis thoracica	Between discrete color morph/sex classifications	Positive	-	-	-	Busso et al. 2017
Lepidoptera						
Galleria mellonella (larvae)	Between geographically distinct melanic vs. non-melanic morphs	Negative	Infected larvae: Positive Uninfected larvae: NS	Positive	Infected larvae: Negative Uninfected larvae: NS	Dubovskiy et al. 2013

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Lepidoptera (continued)						
Danaus plexippus	Between parasitized and unparasitized males vs. females	-	-	NS	-	Lindsey & Altizer 2009
Lymantria monacha	Between discrete color morphs	-	Positive	-	-	Mikkola & Rantala 2010
Lycaena tityrus (pupae)	Between high vs. low altitude populations across two temperatures	-	NS	-	-	Karl et al. 2010
	Within populations and temperature treatments	-	Low altitude, low temp: NS Low altitude, high temp: Positive High altitude, low and high temps: NS	-	-	
Spodoptera exempta (larvae)	Between groups raised in solitary vs. crowded conditions	Positive	-	-	-	Reeson et al. 1998
Spodoptera littoralis (larvae)	Between groups reared on high vs. low dietary protein quality	NS	-	-	Positive	Lee et al. 2008

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Lepidoptera (continued)						
Spodoptera littoralis & *Spodoptera exempta* (larvae)	Between groups raised in solitary vs. crowded conditions	Positive	Positive	-	-	Wilson et al. 2001
Spodoptera littoralis (larvae)	Between families (quantitative genetics)	NS	-	Positive	NS	Cotter et al. 2004a
Spodoptera littoralis (larvae)	Between groups raised in solitary vs. crowded conditions	Positive	Positive	NS	Negative	Cotter et al. 2004b
Spodoptera littoralis (larvae)	Between dark vs. pale selection lines	Negative	-	-	Positive	Cotter et al. 2008
	Within selection lines	Positive	-	-	NS	
Odonata						
Coenagrion puella	Between groups exposed to ultraviolet radiation vs. non-exposed during development	Positive	Negative	NS	-	Debecker et al. 2015
Erythrodiplax funereal	Within individual males	-	Negative	-	-	Contreras-Garduño et al. 2011

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Odonata (continued)						
Paraphlebia zoe	Between discrete morph/sex classifications	Positive	-	-	-	Ruiz-Guzmán et al. 2013
Calopteryx splendens	Within individual males	-	Positive	NS	-	Rantala et al. 2000
Hetaerina americana	Within individual males	Winter: Negative Summer: NS	NS	-	-	González-Santoyo et al. 2010
Hetaerina cruentata		Winter: Negative Summer: NS	-	-	-	
Hetaerina occisa		Winter: NS Summer: Negative	-	-	-	
Hetaerina titia		Winter & Summer: NS	-	-	-	
Hetaerina vulnerata		Winter & Summer: Negative	NS	-	-	
Micrathyria catenata (larvae)	Between individuals that did vs. did not encapsulate an implant	-	NS	-	-	de Oliveira & de Marco Júnior 2009

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	**Encapsulation response**	**Hemocyte counts**	**Antibacterial activity**	
Odonata (continued)						
Calopteryx splendens xanthostoma	Within individual males	Pre-immune challenge: NS Post-immune challenge: Negative	NS	-	-	Siva-Jothy 2000
Orthoptera						
Anabrus simplex	Between low vs. high density morphs	-	Positive	-	Positive	Bailey et al. 2008
Anabrus simplex (nymphs and adults)	Between brown vs. green nymphal morphs	NS	Positive	-	NS	Bailey 2011
	Between brown vs. green nymphal morphs tested as adults	Positive	NS	-	NS	
Hemideina maori	Between discrete morph/sex classifications	-	Negative	NS	-	Robb et al. 2003
Metrioptera roeseli	Between brown vs. green male morphs	-	NS	-	-	Berggren 2010

Species	Experimental comparisons	Immune trait relationship with cuticle darkness				References
		Humoral PO activity	Encapsulation response	Hemocyte counts	Antibacterial activity	
Orthoptera (continued)						
Allonemobius socius	Between groups at two temperatures	Positive	-	-	Negative	Fedorka et al. 2013a
	Within temperature treatments	NS	-	-	NS	
Allonemobius socius	Between geographically distinct populations	Positive	Positive	-	-	Fedorka et al. 2013b
	Within populations	NS	Females: NS Males: Positive	-	-	
Tetrix undulata	Between discrete color morphs at two temperatures	-	NS	-	-	Civantos et al. 2005
Teleogryllus oceanicus	Between groups at three temperatures	Positive	-	-	Positive	Ehrlich & Zuk 2017 (thesis)
	Between sexes	Negative			NS	
	Within sexes	Females (wing): NS Females (leg): Negative Males (wing and leg): NS	-	-	NS	

Appendix III

Figure 4: Average number of days between hatching and eclosion for female, normal-wing male, and flatwing male *Teleogryllus oceanicus* across experimental rearing temperatures.