CLIMATE CHANGE AND THE EVOLUTION OF INSECT IMMUNE FUNCTION

by

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ABSTRACT

Insects are ectothermic organisms that have physiological, behavioral and life-history traits directly influenced by their environment. Investigations have shown that many insects use melanin to permanently darken their cuticles in cooler or drier climates to improve thermoregulation and desiccation resistance. Melanin is a major component of pathogen defense in insects. This suggests that environmentally driven adaptive changes in cuticular melanin may non-adaptively shape insect immune function. This hypothesis has been referred to as climate-related Cuticle Dependent Immune Investment (climate-related CDII).

Climate-related CDII also suggests that a warming climate could lead to the evolution of a weakened melanin-based immune response due to direct selection for lighter cuticles. Climate-related CDII has not been investigated with regard to climate change. Using Drosophila melanogaster, the first part of this study investigated if the documented pattern of lowered immune function in warmer temperatures offsets the expected gain in metabolic rate. The second part of this project investigated how a warming thermal environment will affect the evolution of insect immune function by quantifying changes in melanization and immune function over multiple generations in a changing thermal environment. In the first investigation there was evidence for weakened immune function in males, while females saw an offset by gaining a metabolic boost. The second investigation showed evidence that warming treatments evolved lowered overall immune function. This project gives evidence that insect immune function has the potential to be weakened by increasing temperatures. Insect immune function is a major contributing factor to insect abundances. A decrease in beneficial insects or an increase in harmful insects or pathogens they vector could have detrimental environment and human health consequences.

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INTRODUCTION

Insects are a diverse group of ectotherms that impact multiple facets of global ecology. They are major contributors to ecosystem health, agricultural health and human health, considering that they can induce extensive beneficial or detrimental effects within ecosystems. For instance, beneficial insects can provide essential ecosystem / agricultural services such as pollination and soil aeration, while harmful insects may spread devastating human and agricultural disease (e.g. malaria, citrus greening). Therefore, a decrease in the abundance of beneficial insects, or an increase in the abundance of insect vectors or vectored pathogens, can have detrimental environmental and human health consequences.

An important component in regulating these abundances (and hence environmental/human health) is the insect immune system. The interdependency between insect immunity and human health is well exemplified by Colony Collapse Disorder (CCD); the recent phenomenon occurring within North American populations of the western honey bee, *Apis mellifera*. Honey bees are important for the role they play as pollinators, and have an especially large economic impact stemming from this ecosystem service. CCD is characterized by a rapid loss of life of adult worker bees without a singular known cause (vanEngelsdorp et al. 2009). What is believed to be driving the phenomenon is a combination of factors related to stress and disease. In short, neonicotinoid pesticide exposure appears to induce immune function decline in bees, which makes them more susceptible to diseases such as Deformed Wing Virus (vanEngelsdorp et al. 2009). CCD stands as a prime example of how anthropogenic driven causes (pesticide use) can lead to insect immune system modification, which in turn alters ecosystem / human health by modifying insect abundances. The profound influence insect

abundances have on global ecology makes understanding the natural and anthropogenic factors that drive variation in insect immunity imperative.

A central aspect of insect immune function is the pigment melanin, which helps to defend against a wide range of pathogens including fungi, viruses, bacteria and protists (Nappi and Christensen, 2005). To fulfill its defensive role, melanin based immunity employs two main mechanisms, (i) phenoloxidase activity and (ii) encapsulation. When a pathogen is encountered, the phenoloxidase enzyme is released from circulating granulocytes directly into the hemolymph. Phenoloxidase facilitates the production of melanin by converting tyrosine to melanochrome, which non-enzymatically converts into melanin (True, 2003; **Figure 1**). Small invading organisms such as bacteria are killed by the cytotoxic intermediates involved in melanin production (e.g. reactive oxygen species). If the invading organism is large (e.g. nematodes or parasitoid eggs), hemocytes will surround the invader and release phenoloxidase, which causes the cells to stick together and melanize, encapsulating the invader. This process creates multiple layers of dead melanized hemocytes that isolate and kill the parasite through oxygen deficiency (Gillespie et al. 1997). The production of cytotoxic intermediates also aids in the demise of the encapsulated invader.

Importantly, melanin is also adaptive in cuticle coloration, including camouflage (crypsis), sexual selection, and warning defense (aposematism). The most ubiquitous selective pressure shaping cuticle color, however, is climate. For instance, cuticle color allows insects to more efficiently regulate body temperature, with darker cuticles being favored in cold climates and lighter cuticles being favored in warm climates (Watt 1968). Relative humidity also influences cuticle color, with darker cuticles being favored under drier, low humidity conditions due to its improved desiccation resistance (Parkash 2008). It is important to note that the

production of cuticular melanin and immune melanin use nearly identical biochemical pathway (**Figure 1**). Thus, selection on the cuticle for improved thermoregulation or desiccation resistance could have indirect effects on melanin-based immunity; a phenomenon we have termed climate-related Cuticle Dependent Immune Investment (CDII).

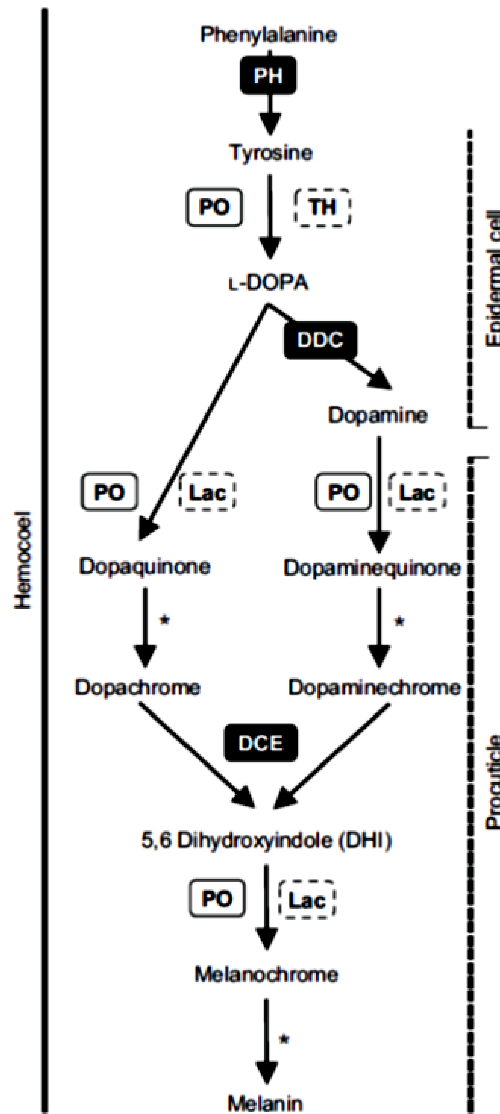

Figure 1- Melanin Production in insects. Immune melanin is occurs in the hemocoel and is facilitated by the enzyme phenoloxidase (PO). Cuticle melanin occurs mainly in the cuticle and is facilitated by the enzyme laccase phenoloxidase (Lac). All enzymes in black are shared between the two pathways. From Kutch et al. 2015.

Climate-related CDII has been investigated from several perspectives and across multiple insect taxa. Fedorka et al. 2013(a,b) investigated this dynamic using *Allonemobius socius*, the southern ground cricket. In this study, cricket populations from warmer climates exhibited lighter cuticles and weaker melanin-based immunity. These population differences persisted when individuals were reared in a common environment, suggesting local adaptation in cuticle color coupled with a maladaptive shift in immune function (Fedorka et al. 2013a). This investigation was expanded to examine seasonal plasticity (Fedorka et al. 2013b). The results of this study suggest that cuticular melanin investment is adaptively plastic within populations; with summer months producing lighter crickets and weaker immunity. Kutch et al. 2014 examined climate-related CDII within a distantly related species of insect, *Drosophila melanogaster*. The findings of this study supported the previous results found in *A. socius* (Fedorka et al. 2013b). Therefore, there is strong evidence that temperature directly shapes cuticle color (Watt 1968; Parkash et al. 2008), which indirectly shapes melanin-based immunity (Fedorka et al. 2013 a,b) and that this phenomenon is evolutionarily conserved across insects groups (Kutch et al. 2014).

Considering the evidence and assumed ubiquity for climate-related CDII, a warming climate might have a devastating impact on agricultural, ecosystem and human health if insects developing in warmer temperatures exhibit a reduced melanin-based immunity infrastructure. This could have a notable impact on pollinators (e.g. bees) by making them more susceptible to disease, thereby disrupting agriculture and natural systems. Rising temperatures should also increase the infection level of pathogens within insect vectors, potentially increasing pathogen transmission to the definitive host (Harvell et al. 2002). Evidence already exists for the impact of a changing climate on insect cuticle melanization. Brakerfield and de Jong found evidence of a decline in melanism in the two-spot ladybird, *Adalia bipunctata* from 1980-2004**.** They attribute

these differences to climate change and local selection (Brakerfield and de Jong 2011). Thus, climate warming over just 24 years has had such a profound impact on melanization of one insect species. Unfortunately, this work did not examine immune function, resulting in a lack of real world information on how climate change might influence insect immune defenses.

As temperatures increase, insect metabolic rates and enzyme kinetics are also expected to increase, which in turn should increase immune defense (Adamo and Lovett 2013). Thus, any decline in the amount of melanin producing infrastructure may be offset by an increase in its efficiency, making the effect of climate change on insect immunity unclear (**Figure 2**).

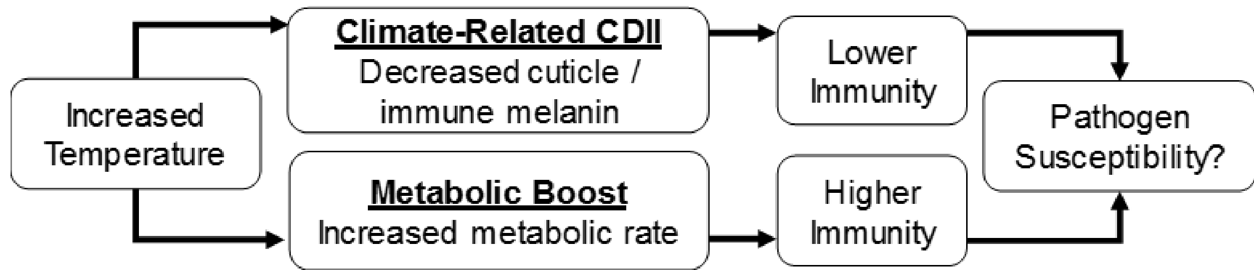

Figure 2- A path diagram representing the opposing pressures on insect immune function expected with an increase in temperature. Climate-related cuticle dependent immune investment expects a drop in immune function when temperature is increased (IF). The opposite is expected with metabolic boost, where insect immune function (IF) should be increased due to the increase in temperature.

This uncertainty is further compounded when one considers how the virulence of insect pathogens are also affected by temperature (e.g. warmer temperatures may either impede or promote pathogen growth and virulence). Pathogen virulence aside, it is still important to know if insect hosts can compensate for a reduced melanin-based immune response though a temperature induced increase in metabolic rate. If true, then warming climates may lead to the evolution of insects with improved defensive capabilities.

D. melanogaster has been shown to follow Bergman's rule of body size. Flies in cooler latitudes and elevations have larger body size (Partridge et al. 1994). The temperature influenced

difference in body size could be potentially interacting force acting upon differences seen in immune response. While this is something that would be difficult to untangle, it does not take away from answering the global hypothesis of temperature's effect on insect immune function. If body size differences are driving a portion of the variation does not change the interpretation of temperatures effect on insect immune function.

Here I assessed two hypotheses regarding insect immunity and temperature. The first hypothesis centers on phenotypic plasticity and states that warming temperatures will increase immune function (presumably via metabolic rate increase and increased enzyme kinetics), but will not offset the overall decline in immune function due to a reduced investment in melanin-based immune infrastructure (**Figure 3**).

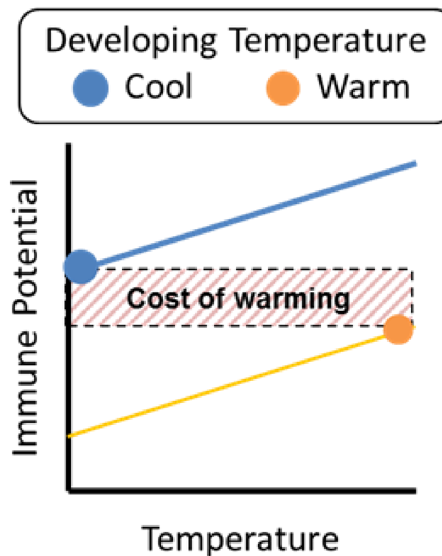

Figure 3- As temperatures warm, insects that developed in both cool and warm environments improve their immune potential via a metabolic rate increase or increased immune enzyme efficiency (increased slope). However, warm developing insects invest less in melanin producing infrastructure in order to optimize thermoregulatory needs (decreased intercept). This means that as temperatures warm, the benefit gained from a metabolic rate increase might not compensate for the reduction in lower melanin-producing infrastructure.

The second hypothesis centers on evolutionary / genetic adaptation and states that the predicted increase in temperature of the next 100 years will select for populations with lighter cuticles and a weakened melanin-immune system infrastructure. To assess these hypotheses, I used the fruit fly model system, *Drosophila melanogaster*. This organism was chosen because of its utility in multigenerational laboratory selection studies, and its well characterized immune system. In short, we predict that warming temperatures will lead to the development (hypothesis 1) and evolution (hypothesis 2) of insects with a reduced capacity for melanin-based immune defense, becoming more susceptible to pathogenic infection.

METHODOLOGY

Hypothesis One

As previously stated, work in both *D. melanogaster* and *A. socius* suggest that insects developing in warm summer-like conditions exhibit reduced melanin-based immune investment (i.e. a reduced amount of melanin and melanin-producing capacity in the cuticle and hemocoel). Specifically, insects developing under summer-like conditions (28.5°C 14:10 L:D) were found to possess less melanized cuticles and a greater susceptibility to pathogenic infection compared with their counterparts developing under fall-like conditions (21.5°C 10:14 L:D). Importantly, immune function in these studies was assayed under common conditions (25°C 12:12 L:D). Although this approach allowed for immune infrastructure differences to be detected between the treatment conditions, it controlled for differences in metabolic rates. It may be that the increased metabolic rate inherent in warmer temperatures would provide "summer" insects an equal or greater capacity to deal with pathogenic threats compared with their "fall" counterparts. In other words, the boost in metabolism gained by "summer" insects may make their immune system more efficient, thereby compensating for their lower level of immune infrastructure.

Experimental Design - To address my first hypothesis, flies were reared at two temperature treatments (21.5ºC and 28.5ºC) in standard 200 ml fly bottles using Percival incubators and a standard light cycle (12:12 L:D). The parental population came from an outbred laboratory stock that was collected in Orlando in the summer of 2013 and maintained at 25ºC. To generate the flies needed for this hypothesis, 15 mating pair of 3 day old virgin flies were placed into a 200ml bottle and allowed to mate for 48 hours. After mating, females were transferred to a fresh bottle for oviposition. After 24 hours the females were removed and the oviposition bottles were then randomly placed into either the 21.5ºC or 28.5ºC temperature treatment. At adult eclosion, flies

were separated by sex and randomly assigned to have their immune system assayed at either 21.5°C or 28.5°C. This set up yielded two distinct rearing temperatures, and two distinct assaying temperatures. This gave a total of four combinations of rearing and assay temperature (Cool:Cool, Cool:Warm, Warm:Cool, and Warm:Warm). Rearing and assay temperatures were treated as individual treatments. At 3 days (+/- 1) after emergence, the flies were assayed for their cuticle melanization and two aspects of immune function, including phenoloxidase activity, lytic activity (**Table 1**). It is important to note that host bacterial resistance (i.e. assaying host survival against a virulent pathogen) was not assayed, as this assay added a host independent variable, the pathogen. Considering that the effect pathogens have on their hosts in warmer climates depends entirely on the pathogen, I limited my examination of immune potential on the host only.

Table 1- Assays used to address hypothesis 1

Assay	**Variable Tested**	**Experimental Unit**	**Sample Size**
Phenoloxidase Activity	Melanin-based Immunity	Hemolymph sample (30 flies per sample)	48 per sex; per treatment **Total = 384 (rep = 3)**
Lytic Activity	Non-Melanin-based Immunity	Hemolymph sample (30 flies per sample)	48 per sex; per treatment **Total = 384 (rep = 3)**
Cuticular Melanization	Cuticle Darkness	Individual flies	80 per sex; per treatment **Total = 640 (rep = 3)**

Phenoloxidase and Lytic activity – To estimate the concentration of phenoloxidase (melanin-based immunity) and lysozyme-like enzymes (non-melanin-based immunity) in the hemocoel, we estimated PO and lytic activity in hemolymph. PO activity measures the rate at which melanochrome is created when a hemolymph sample is seeded with the PO enzymatic substrate L-Dopa (**Figure 1**). Lytic activity measures the rate at which bacterial cells are lysed when a hemolymph sample is seeded with the gram-positive bacteria *Micrococcus luteus*. For PO

activity, this means that the reaction vessel containing hemolymph and L-Dopa will darken over time, while for lytic activity a reaction vessel containing hemolymph and *M. luteus* will lighten over time.

In order to collect hemolymph for each immune assay, 30 flies were pricked with a fine tipped needle and placed in a 0.5ml microcentrifuge tube containing a small hole in the base (the hole was created using a 25 gauge needle). The 0.5ml tube was then placed into a larger 1.5ml tube and 20µl of phosphate buffered saline (PBS) was added. Each tube was centrifuged at 8000g for 5 minutes to pull hemolymph and PBS into the lower collection tube. The hemolymph samples were then stored at -80°C until analyzed.

Samples were read for optical density, using flat-bottomed 96 well plates on a Bio Rad Model 680 micro palte reader, at both 490 and 450 nm. Each well was loaded with 20 µl of stored hemolymph/PBS sample. Samples being assessed for PO activity had 14 µl of α-chymotrypsin was added. α-chymotrypsin is a serine protease, and works to cleave prophenoloxidase (PPO) and allow for phenoloxidase activity and melanochrome production (Lu et al. 2014). Wells designated for the lytic assay were also loaded with 20 µl of stored hemolymph/PBS samples and an additional 14 µl of PBS was added to maintain consistent volume with PO assay wells. After the addition of α-chymotrypsin and PBS, the plate was allowed to rest for 20 minutes before addition of regents. L-DOPA was added (90µl) to PO designated wells and *M. luteous* was added to lytic wells. The plate was then inserted into the microplate reader. Optical density of samples was recorded at 10 minute intervals for 90 minutes using the BIORAD microplate reader. Every other data collection point (10, 30, 50, 70, and 90) occurred after 10 minutes of programmed shaking in the microplate reader to ensure bacteria was

not settling at the bottom of the well. Phenoloxidase (PO) assays were read at 490nm and lytic activity was assessed at 450nm.

Cuticle melanization – In order to estimate the amount of melanin in the cuticle, we used a photographic assay that provided a grayscale measure of cuticle darkness. A subset of individuals from each treatment were placed on a microscope slide in groups of 40. Flies were imbedded in liquid hand sanitizer (Purell) to maintain proper orientation for the camera. Photographs were taken using BK Plus Lab Imaging System (Visionary Digital). This program standardized lens height, zoom, and flash in high resolution pictures. This standardization allowed for a comparison of average pixel darkness. From each picture, an individual's pronotum and abdomen darkness was measured using ImageJ by estimating its mean grayscale value. Pronotum length and head width were used to as an estimate of body size.

Statistical Analysis- To address the first hypothesis, we employed mixed model nested ANOVA. To determine if treatments differed in cuticle darkness and body size, rearing temperature and sex were fixed factors (**Table 2**). The random variables were experimental replicate and photographic slide nested within replicate. To determine if immune function differences existed, rearing temperature, assay temperature and sex were fixed factors with experimental replicate and reaction plate nested within replicate as random variables (**Table 2**). Where appropriate, a principle component analysis (PCA) based on covariance was used to create an overall "cuticle darkness" factor and a "body size" factor (**Table 2**). These factors were based on the first principle component of thorax darkness and abdomen darkness, as well as head width and thorax length, respectively.

Table 2- Table showing variables and statistical models for hypothesis 1.

Assay	Investigation	Sample Size	Model # denotes random effect
Phenoloxidase Activity	Melanin-based Immunity (MBI)	48 per sex; per treatment **Total- 384**	Y= Δ90 = PO (90) y= PO (90) = rear + assay + #rep + #plate[rep] **BY SEX**
Lytic Activity	Non-MBI	48 per sex; per treatment **Total- 384**	Y= Δ90 = Lyt (90) y= Lyt (90) = rear + assay + #rep + #plate[rep] **BY SEX**
Cuticular Melanization (+ Body Size)	Cuticle Darkness (+ Body Size)	80 per sex; per treatment **Total- 640**	PC1-Cov = rear + sex + rear*sex + #Rep + #slide[rep] PC1 – CM = Darkness (Thorax + Abdomen) PC1 – BS = Body Size (Head Width + Pronotum Length)

Hypothesis Two

Experimental Design - To address this hypothesis, we adopted a laboratory selection approach. Laboratory selection is different from artificial selection in that fly phenotypes were not directly selected. Instead, environmental conditions were established and populations were allowed to evolve along unforced phenotypic trajectories. To this end, three different temperature regimes were established using Percival incubators (model I36-VL), including (i) seasonal warming (S), (ii) aseasonal warming (A), and (iii) static control (C). In both the seasonal and aseasonal warming treatments, temperatures were incrementally raised (~ 0.25°C) every other generation for 28 generations from July 2010 temperature conditions to July 2110 temperature conditions. Thus, every odd-numbered generation (i.e. 1, 3, 5, etc) represented a 7.5 year increase in time. The future predicted temperatures were based on the CMIP5 climate model (IPCC 2013), which predicted a 3.06°C increase in July daytime highs (33°C – 36.03°C) and a 3.18°C increase in July nighttime lows (24°C – 27.12°C) between 2010 and 2110 . The seasonal treatment was the same

as the aseasonal treatment, except that each generation alternated between predicted July and October temperature conditions (October daytime highs are predicted to increase 3.3ºC (29ºC – 32.34) while nighttime lows are predicted to increase 3.99ºC (20ºC – 24ºC)). To keep both populations temporally synchronized, the aseasonal warming treatment conditions were kept unchanged during seasonal treatments October cycle. The static control treatment was maintained continually at the 2010 starting temperature conditions during the duration of the experiment (Daily Cycle between 24ºC and 33ºC) (**Figure 4**).

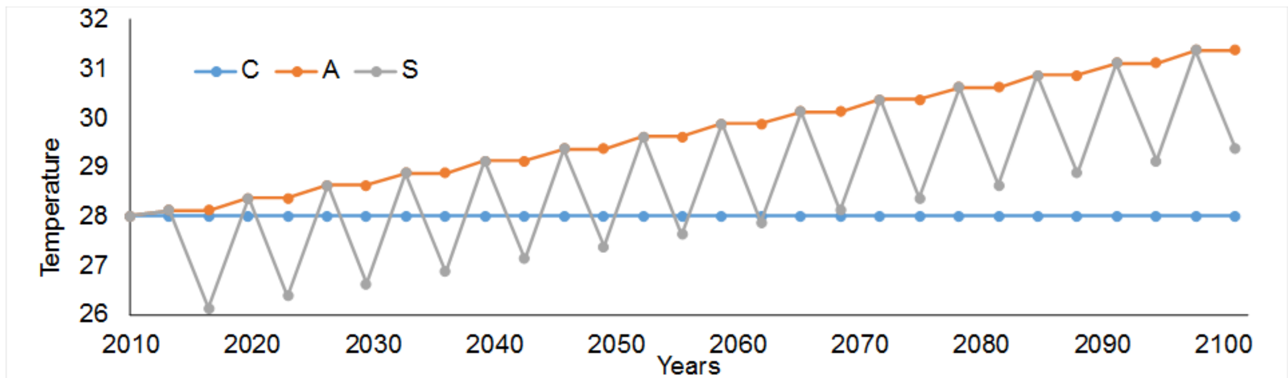

Figure 4- The treatments. The control treatment (C) maintain a constant temperature throughout the period of the experiment. The aseasonal treatment (A) increased 0.25°C every other generation. The seasonal treatment (S) fluctuated between summer and fall temperatures, and each generation experienced an increased in fall and summer temperatures. Each data point represents a generation. This figure does not include the daily cycle variation around a daily mean that each treatment experienced

The motivation behind using both a seasonal and aseasonal treatment is simple. In most systems, animals will experience seasonal temperatures that include hot summers, cool falls / springs, and cold winters. If we select insects under a warming environment without accounting for this seasonal change, we may dramatically overestimate the evolutionary response. This experimental design allowed us to address two additional hypotheses. The first hypothesis is that seasonality will retard evolutionary response due to divergent cyclical selection. This temporal "tug of war" over the optimization of phenotypes predicts that aseasonal populations will evolve lighter cuticles faster than will the seasonal populations. The second hypothesis is that

seasonality promotes gene by environment interactions, inducing greater phenotypic plasticity. This creates two predictions: (1) seasonal flies will possess higher fitness across the summer and fall environments compared with aseasonal flies, and (2) aseasonal flies should be more highly canalized due to experiencing a more stable environment through time. Here we address the latter prediction and expect that flies from the aseasonal treatment will have a lower coefficient of variation compared with their seasonal counterparts.

To more accurately simulate natural daily cycles, temperatures increased between midnight and midday and decreased between midday and midnight along a sine wave pattern (**Figure 5**). The daily high and low temperatures for each generation were determined by taking a mean of the predicted daily highs and lows for the month of the treatment.

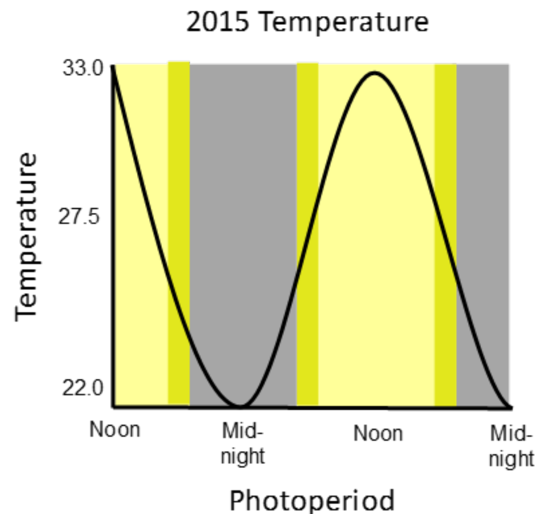

Figure 5- Daily light and temperature cycle. Temperature followed a sine wave pattern. Additional lights (bright yellow) turned on 2 hours after the incubator lights turned (dark yellow) on and they turned off 2 hours before the incubator lights turned off to simulate lower light levels during dusk and dawn.

Lights also simulated a daily cycle, with the incubator lights turning on and off according to the seasonal photoperiod (14:10 L:D for July and a 11:13 L:D for October). Each treatment incorporated an additional light source (Growbright T5) to increase lux within treatments to that

of an average overcast/sunny day (30,000 lux). The additional lights turned on 2 hours after the incubator lights turned on and they turned off 2 hours before the incubator lights turned off to simulate lower light levels during dusk and dawn. Treatments were randomly rotated across three incubators to minimize incubator effects.

Fly stock and maintenance - The fly stocks used in this experiment were from a wild stock caught in Orlando, FL during summer 2013. This wild stock had been previously reared at a constant temperature of 25°C, and kept on a strict 14 day generation cycle. For the purpose of the experiment, flies were also maintained on a strict 14 day generation cycle. All flies were kept in population boxes (18cm X 18cm X 19cm) that housed four food bottles. At day 12 in the generational cycle, food bottles were replaced with fresh unsealed food bottles and the fly population was allowed to mate freely and oviposit. At day 14, the oviposition bottles were sealed and the adult population was culled. At day 10 of the new generation, the bottles were unsealed and the next generation of flies allowed to emerge into the population box. Prior to experimentation, the fly stock was maintained under the control conditions (fluctuating light cycle and average temperature of 28°C for four generations. After this time, the stock was randomly divided into their treatments, with four replicate populations per treatment.

Experimental Assays - At generation 28, flies from each treatment were assessed for their cuticle melanin, phenoloxidase activity and lytic activity as before (**Table 3**). In addition, flies were also assayed for their ability to resist a bacterial infection. To assay bacterial resistance percent mortality was used as an index of population immune function. This was accomplished through prick infections on flies with the gram-negative bacterium *Pseudomonas aeruginosa*. A LD50 concentration was used to infect individuals using a fine tipped needle (Fine Science Tools

model # 26002-20). The flies were checked for survival at 24, 48, and 72 hours post infection. Flies were kept at a density of (n=5) for the duration of infection.

Table 3 – Assays used to address hypothesis 2

Assay	Variable being tested	Experimental Unit	Sample Size
Phenoloxidase Activity	Melanin-based Immunity	Hemolymph (30 flies per sample)	32/sex/treatment/population **Total- 768**
Lytic Activity	Non-Melanin-based Immunity	Hemolymph (30 flies per sample)	32/sex/treatment/population **Total- 768**
Cuticular Melanization	Cuticle Darkness	Individual flies	32/sex/treatment/population **Total- 1152**
Bacterial Resistance	Population Immunity	Individual flies	32/sex/treatment/population **Total- 2400**

Statistical Analysis- The second hypothesis was analyzed similar to the first hypothesis. To avoid incubator effects, the experiment was replicated 4 times, with the treatments switched between incubators with each replicate. For cuticle darkness and body size, treatment, population nested within treatment and sex were fixed variables, with replicate and slide nested within replicate as random variables. For immune function, treatment, population nested within treatment and sex were fixed, with replicate and reaction plate as random variables (**Table 4**). To assess bacterial resistance, we employed a general linear model using the binomial distribution (Logit link function), with treatment, population nested within treatment and sex as fixed variables, and replicate and fly vial nested within replicate as random variables. Cuticle darkness and body size factors were also constructed via PCA (**Table 4**). All analyses were conducted in JMP version 12 Pro (SAS Institute, Cary, North Carolina). Post hoc Tukey's HSD tests were used to assess differences between the treatments following analysis.

Table 4 - Table showing variables and statistical models for hypothesis 2

Assay	**Investigation**	**Sample Size**	**Model** **# denotes random effect**
Phenoloxidase Activity	Melanin-based Immunity (MBI)	24 per sex; per treatment; per population **Total- 576**	Y= Δ90 = PO (90) y= PO (90) = trt + pop[trt] + #rep + #plate[rep] **BY SEX**
Lytic Activity	Non-MBI	24 per sex; per treatment; per population **Total- 576**	Y= Δ90 = Lyt (90) y= Lyt (90) = trt + pop[trt] + sex + sex*trt + #rep + #plate[rep]
Cuticular Melanization (+ Body Size)	Cuticle Darkness (+ Body Size)	36 per sex; per treatment; per population **Total- 864**	Thorax= trt + pop[trt]+ #rep + #slide[rep] PC1-Cov = trt + pop[trt] + #rep + #slide[rep] CM = Thorax PC1 – BS = Body Size (Head Width + Pronotum Length)
Bacterial Resistance	Realized Immunity	**75 per sex; per treatment** **Total- 1800**	Y= Mort 72 = % Mortality at 72 Hours y= Mort 72 = trt + pop[trt] + sex + sex*trt + #rep + #vial[rep]

RESULTS

Hypothesis One

In general, females were bigger (mean body size PC1 score ± se: 3.8 ± 0.2 versus -3.8 ± 0.2, respectively; $P < 0.0001$) and darker than males (mean darkness PC1 score ± se: 2.2 ± 0.5 versus -2.35 ± 0.5, respectively; $P < 0.0001$). Body size estimates were based on the first principle component of head width and thorax length, while darkness estimates were based on the first principle component of thorax and abdomen cuticle darkness. PC1 mean estimates were based on covariances and were controlled for rearing temperature, replicate and photographic slide. In addition, rearing temperature had a significant effect on body size and cuticle darkness in both sexes (**Figure 6** and **Table 5**). The statistical interaction between rearing temperature and sex was due to males responding to rearing temperature more than females. These data indicate that rearing temperature produced a robust effect on male and female physiology.

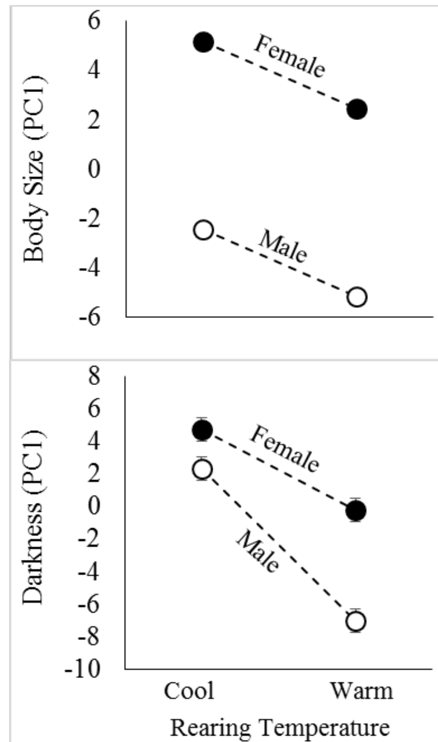

Figure 6 - Effect of rearing on body size and cuticle darkness. Warmer rearing temperature resulted in smaller, lighter flies. Points represent means + se (body size se too small to see).

With regard to immunity, rearing temperature had an effect on phenoloxidase activity in both sexes (**Figure 7**, **Table 5**), with cold-reared insects exhibiting a greater activity. This result is expected, considering that colder reared insects have been shown to invest more in melanin producing infrastructure, which includes the phenoloxidase enzyme. In addition, PO activity was positively influenced by a warmer assay temperature in females. This result was also expected, considering that warmer temperatures can improve enzyme kinetics, which increases the rate at which phenoloxidase converts L-DOPA into melanochrome. Importantly, the PO activity of warm-reared, warm assayed females exceeded the PO activity of their cold-reared, cold assayed counterparts (0.72 ± 0.06 versus 0.47 ± 0.06, respectively; $F_{1,83} = 7.92$, $P = 0.0061$; **Figure 7a**), indicating that females did not suffer a PO activity cost due to developing in a warmer environment. Interestingly, males did not exhibit an increase in PO activity with increased assay temperature and the PO activity of cold-reared, cold assayed males exceeded that of their warm-

reared, warm assayed counterparts (0.60 ± 0.05 versus 0.43 ± 0.05, respectively; $F_{1,83} = 15.97$, $P < 0.0001$; **Figure 7a**). Thus, it appears that as temperatures rise (e.g. seasonally), improved phenoloxidase efficiency in females compensates for reduced phenoloxidase levels. However, males appeared to suffer an immune cost in warmer temperatures due to an under investment in melanin-based immunity.

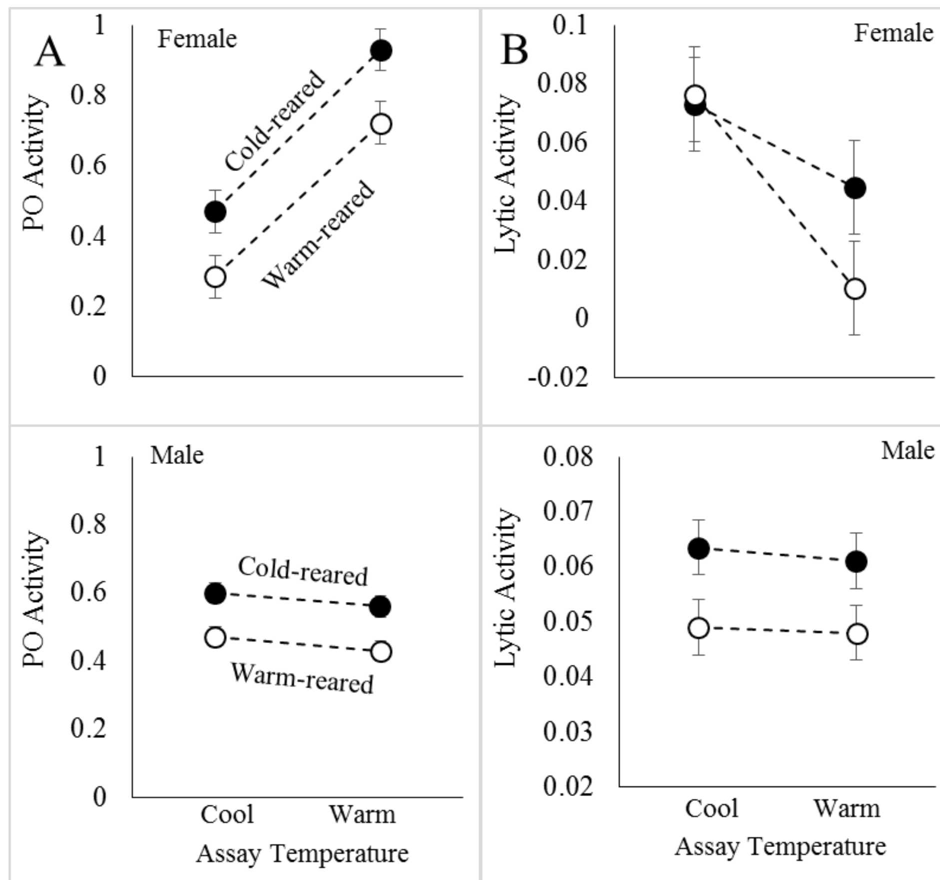

Figure 7 - Effect of rearing and assay temperature on immune potential. (A) Insects reared in colder environments exhibited a higher phenoloxidase activity. Warmer assay temperature improved PO activity in females, resulting in a net increase in PO activity in warmer environments. (B) Females exhibited no effect of rearing temperature on lytic activity, but exhibited a decline in activity in warmer assay temperature. Cold reared males exhibited a greater lytic activity, but showed no effect of assay temperature.

Rearing temperature did not influence lytic activity in females, which was expected considering that lysozyme is not directly related to melanin-based immune function (**Figure 7b, Table 5**). As assay temperature increased, female lytic activity declined. This result may be due

to the lyophilized *M. luteus* actively dividing in the reaction well, whose rate of cellular division exceeded the rate of cellular lysis at the higher assay temperature. As with phenoloxidase activity, males reared at cold temperatures exhibited a stronger lytic activity, but assay temperature did not appear to influence the rate of cellular lysis.

Table 5 - Hypothesis 1 statistical analysis. Asterisk (*) denotes random effects.

Source	DF Num	DF Den	F	P<
Body Size (PC1)				
Both Sexes				
Rearing Temp	1	620	89.8	0.0001
Sex	1	620	707.9	0.0001
Rear X Sex	1	620	0.001	0.9736
Rep *	3	12	162.4	0.0001
Slide[Rep] *	12	620	1.535	0.107
Cuticle Darkenss (PC1)				
Both Sexes				
Rearing Temp	1	620	103.5	0.0001
Sex	1	620	47.86	0.0001
Rear X Sex	1	620	9.13	0.0026
Rep *	3	12	784.8	0.0001
Slide[Rep] *	12	620	1.713	0.0601

Source	DF Num	DF Den	F	P<
PO Activity				
Female				
Rearing Temp	1	177	11.9	0.0007
Assay Temp	1	177	51.4	0.0001
Rep *	3	8	12.66	0.0002
Plate [Rep] *	8	177	1.021	0.422
Male				
Rearing Temp	1	177	22.1	0.0001
Assay Temp	1	177	0.9	0.3377
Rep *	3	8	1.14	0.387
Plate [Rep] *	8	177	6.635	0.0001
Lytic Activity				
Female				
Rearing Temp	1	177	0.8	0.3358
Assay Temp	1	177	7.8	0.0058
Rep *	3	8	0.145	0.9301
Plate [Rep] *	8	177	2.259	0.0253
Male				
Rearing Temp	1	177	5.9	0.0161
Assay Temp	1	177	0.1	0.7765
Rep *	3	8	0.26	0.8526
Plate [Rep] *	8	177	13.57	0.0001

Hypothesis Two

After 28 generations, individuals from the control, aseasonal and seasonal treatments were reared for 1 generation in a common environment in order to elucidate any genetic changes in body size and cuticular melanization that occurred. We found that body size had significantly decreased in both the seasonal and aseasonal treatments relative to the control treatments. Thorax cuticular melanin decreased in the aseasonal treatment for both sexes (**Figure 8, Table 6**), but did not decrease in the seasonal treatment.

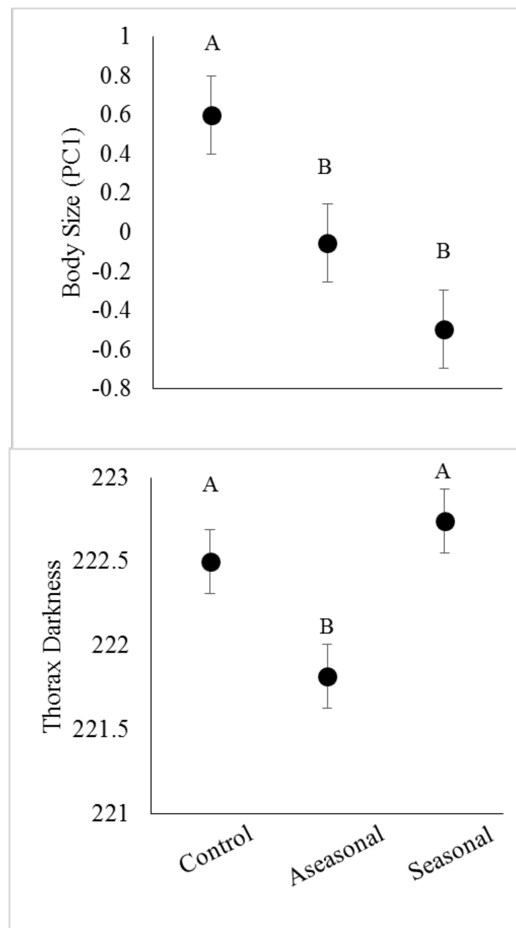

Figure 8 - Effect of laboratory selection on body size and thorax cuticle darkness. The climate change treatments exhibited smaller body size. However, only the aseasonal treatment resulted in lighter cuticles. Treatments connected by the same letter are not significantly different.

This supports the hypothesis that warming climates could lead to insects with lighter cuticles. It also supports the hypothesis that seasonality will somewhat "buffer" an insect's response to warming temperatures. We also found that, contrary to expectation, flies from the seasonal

treatment had a lower coefficient of variation compared with the aseasonal treatment and control (mean ± se: 0.027 ± 0.002 versus 0.35 ± 0.002 and 0.035 ± 0.002, respectively; $F_{2,18} = 5.74$, $P = 0.0118$; estimates of cv were generated for each population and controlled for sex, replicate and photographic slide).

Table 6 - Hypothesis 2 statistical analysis. Asterisk (*) denotes random effects.

Source	DF Num	DF Den	F	P<
Body Size (PC1)				
Both Sexes				
Treatment	2	832	8.2676	0.0003
Population [Trt]	9	832	0.8419	0.5775
Sex	1	832	1289.4	0.0001
Rep *	2	15	162.46	0.0001
Slide [Rep] *	15	832	4.3438	0.0001
Cuticle Darkenss (PC1)				
Both Sexes				
Treatment	2	834	5.9	0.0029
Population (Trt)	9	834	1.26	0.2509
Sex	1	834	320.46	0.0001
Rep *	2	15	70.111	0.0001
Slide [Rep] *	15	834	30.201	0.0001

Source	DF Num	DF Den	F	P<
PO Activity				
Female				
Treatment	2	265	4.7	0.0095
Population (Trt)	9	265	1.3	0.258
Rep *	2	9	1.38	0.2996
Plate [Rep] *	9	265	10.152	0.0001
Male				
Treatment	2	265	1.25	0.2891
Population (Trt)	9	265	0.539	0.8344
Rep *	2	9	0.541	0.6
Plate [Rep] *	9	265	6.794	0.0001
Lytic Activity				
Both Sexes				
Treatment	2	550	0.5	0.6306
Population (Trt)	9	550	1.8	0.0794
Sex	1	550	23.3	0.0001
Sex X Treatment	2	550	2.6	0.0731
Rep *	2	9	0.0257	0.9747
Plate [Rep] *	9	550	7.96.79 4	0.0001

With regard to immune function, there was a significant interaction between climate treatment and sex, resulting in a separate analysis for both sexes. Females exhibited a significant decline in PO activity for both the seasonal and aseasonal treatments, indicating an evolutionary immune cost to increasing temperature over time (**Figure 9, Table 6**). Males exhibited no change in PO activity (**Appendix**). With regard to lytic activity, no effect of treatment was detected (**Figure 9, Table 6**). There was no difference in resistance to bacterial infection across

the treatments (**Appendix**, P < 0.3295). These data suggest that climate change conditions can lead to the suppression of melanin-based insect immune function in females.

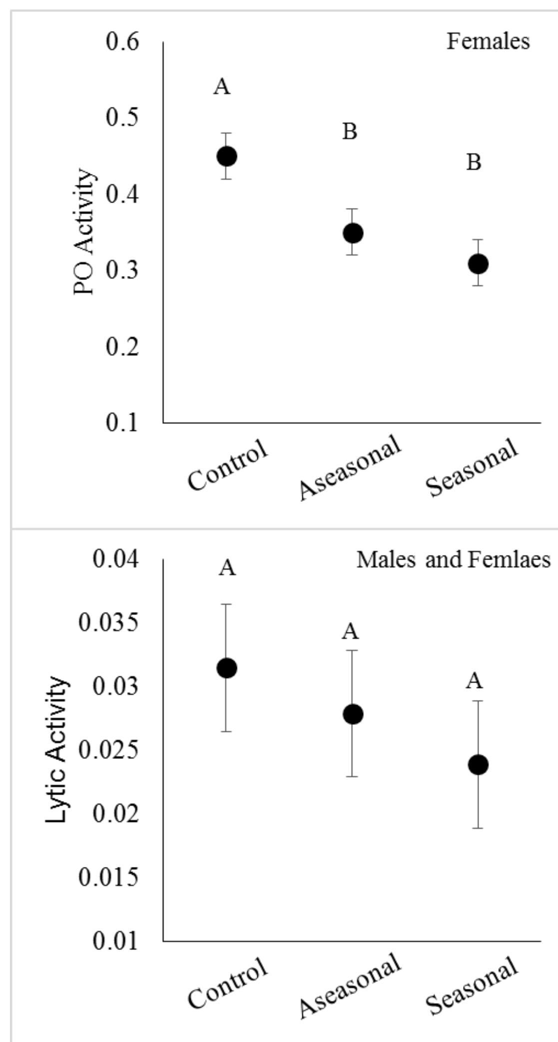

Figure 9 - Effect of laboratory selection on immune potential. The climate change treatments exhibited a reduction in both PO and lytic activity. Treatments connected by the same letter are not significantly different.

DISCUSSION

Understanding how parasite-host interactions may be altered by a changing climate is an important field of study. This is particularly true for insect hosts, considering that insects play vital roles in environmental health and disease prevalence, and their immune function appears to be sensitive to ambient temperature (Fedorka et al., 2013a,b; Kutch et al., 2015). Here we examined how insect immune potential responds when insects develop in, and evolve under, warm environments. Specifically, we addressed how insect immune potential responded to seasonal changes in ambient temperature, as well as how insect immune potential responded to a multigenerational increase in temperature similar to that expected under climate change.

Previous work indicated that insects developing in warm environments invest less in their cuticle melanin and melanin-based immune function, potentially making them more susceptible to pathogenic infection (Fedorka et al., 2013a,b; Kutch et al., 2015). However, with increased temperature comes an increased rate of enzymatic activity for many immune enzymes, making it difficult to predict how pathogen susceptibility will change as temperatures warm (Adamo and Lovett 2013). Considering that a significant drop in melanin producing infrastructure has been detected over just a few degrees Celsius (Fedorka et al., 2013a,b), we hypothesized that increased enzyme efficiencies will not compensate for this decline (**Figure 2**). As with previous studies, we found that insects that developed in warmer environments exhibited lighter cuticles, lower phenoloxidase activity and lower lytic activity. However, females reared and tested in warm environments (i.e. temperatures where warm-reared insects would encounter a pathogen) had a higher phenoloxidase activity than those reared and tested in cold environments (**Figure 7**), indicating that increased enzyme efficiency can compensate for reduced phenoloxidase investment in females. This compensation was not seen in lytic activity or in males. Thus, for

pathogens whose virulence does not increase with increasing temperature, females developing in warm environments should exhibit greater pathogenic resistance. Males, however, should become more susceptible.

With regard to climate change, we hypothesized that predicted warming temperatures will result in less melanized cuticle and weakened insect immunity. After maintaining flies for 28 generations in either a seasonally warming environment, an asesonally warming environment or a non-warming environment (control), we found that smaller, lighter colored flies evolved in the asesonal environment, which is consistent with the plastic response seen in the first experiment. Although smaller flies evolved in the seasonal environment, their cuticle melanin was not different than the controls. This result is likely due to seasonal shifts in temperature retarding selection for lighter cuticles in both fall and summer environments.

With regard to immune potential, females in warming environments evolved a decrease in phenoloxidase activity, while both sexes evolved a decrease in lytic activity. These data suggest that insects will evolve weakened immune potential as climates warm. Why then, did we not detect differences between our treatments with regard to *P. aeruginosa* resistance? Most likely, the LD50 dose used here provides too crude of a tool to uncover fitness-related differences between our warming treatments and controls. Perhaps a more refined measure would be to assess the effects of a sub-lethal dose on male reproductive success or female fecundity. Thus, important differences in pathogen susceptibility may still exist, however, we were unable to detect them in the P. aeruginosa experiment, though PO and lytic activity indicate responses.

If warming temperature will have a negative effect on insect immune function, the frequency and historic range of insect vectored diseases could shift and/or expand due to shifts in

insect susceptibility. There are also implications about how this dynamic might play a role in systems involving plant-pathogen vectors and agricultural pests. Agricultural pests are one of the greatest threats to the world food supply (Rosenzweig et al. 2001; Galli et al. 2009). A potential increase in pathogen prevalence and transmissibility could have major effects on human systems. Pollinators could also be shown to exhibit this relationship of lowered immune function in warmer environments. Pollinator decline due to Colony Collapse Disorder has been widely publicized and investigated within recent years (Galli et al. 2009). If it can be shown that melanin-based insect immune function may be compromised with warming thermal environment, continued investigation would be of immediate importance.

Here, males and females exhibited differences in immune function. In the metabolic boost investigation, looking at a plastic response, assay temperature influence immunity for females and not males. In the adaptive study looking at climate change, only female melanin-based immunity (PO) responded to selection. In general, differences in male and female physiology is driven by the different ways in which the sexes maximize fitness (Bateman 1948). Females tend to invest in maximizing the number of offspring produced, whereas males tend to maximize the number of mating events. This tends to select for long lived females who would be expected to invest more heavily in immunity, and short lived males who tend to invest more heavily in reproduction. This difference in immune investment is commonly referred to as Bateman’s immune principle (Rolff 2001). Therefore, the lack of male response in both the metabolic boost and climate change studies could have been driven by the difference in life history strategy. That is to say, female immunity is more sensitive to changes in temperature than is male immunity.

Given our results, what have we learned about insect immunity and seasonality in a changing climate? Initially, our expectation was that seasonal warming and climate warming would both lead to a weakened insect immune potential. While we did find this for the climate change project in terms of reduced immune-melanin investment, the metabolic boost project showed that females can compensate for the reduced investment. This complicates the interpretation of how females should physiologically respond to climate change. In other words, will females be able to metabolically compensate for the reduced immunity seen via climate change? We are currently addressing this question. Perhaps the only way to glean insight into the future of insect immunity and disease risk in a changing climate is to investigate specific host-pathogen systems, given that the pathogen remains an important variable that was not examined here.

This research has the potential for public education programs focusing on global climate change. It is important to communicate and educate the public on ecosystem-wide effects of global climatic shifts. There are many factors to consider when approaching the evolutionary implications when incorporating such a far-reaching impact as changing climate into such a variable system. This study served as a proof of concept investigating the potential for global consequences related to climate-dependent CDII. An important next step when looking ahead to future investigation would be investigating climate-related CDII across additional insect taxa. Import investigations would include pollinators and disease vectors (**Figure 10**). It is also important to continue investigation into this dynamic from a pathogen perspective. Seeing how various host-pathogen interactions are affected would be an extremely important area of future research. This study can be expanded to include a multitude of different pathogen types (viruses,

fungi, protists, and plasmodium). Future work in this system should broaden the scope of the climatic variables to investigate specific areas of potential concern (**Figure 10**).

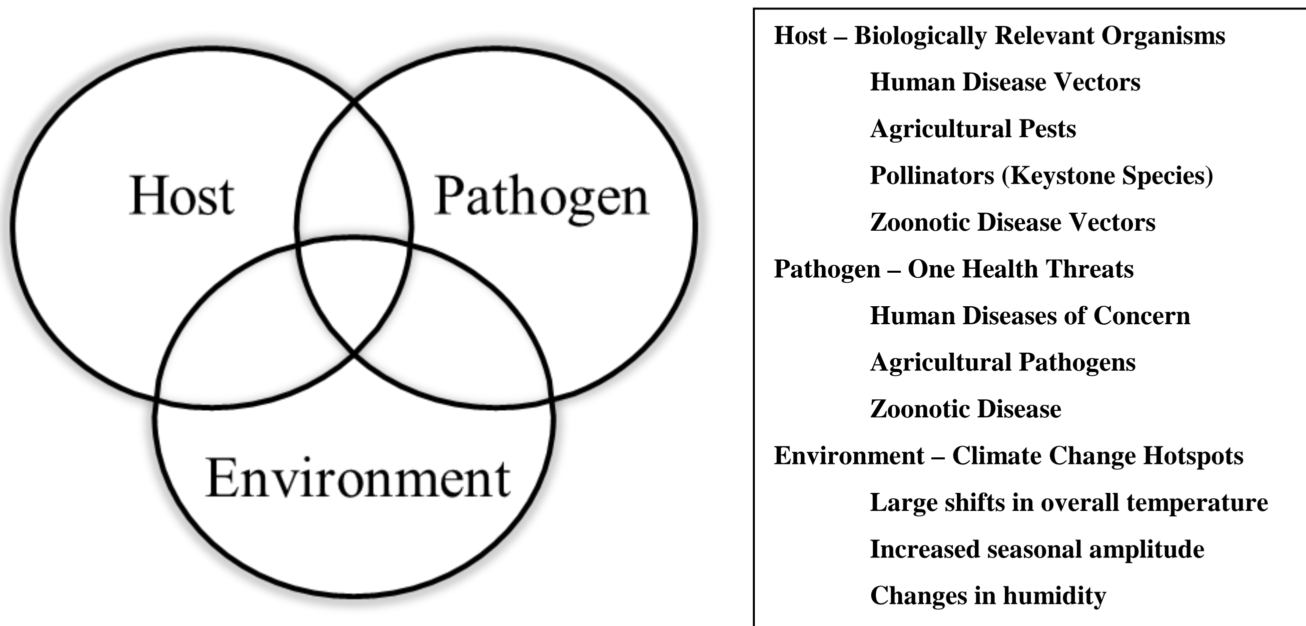

Figure 10 - Future work should focus on the potential for change in insect immunity within specific host/pathogen/environment systems. Areas of concern can be highlighted based on species of interest, disease threat, or expected change in climate.

This study focused on temperature, but humidity may be playing a more an important role as well. Future investigations should focus on areas of the world that are expected to have the largest climatic shifts and large changes in seasonal amplitude. Looking forward, future research should consider specific host, pathogen, and environment systems when investigating further implications of climate change on insect immune function.

APPENDIX: SUPPLEMENTARY MATERIAL

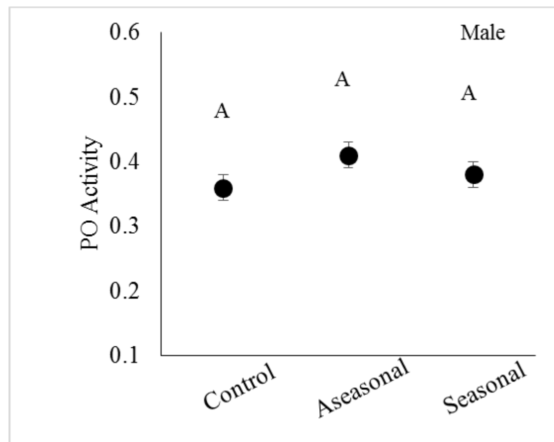

Figure (Appendix 1) - Figure showing results of PO activity assay for hypothesis 2 in males.

Table (Appendix 1) - Table showing statistical analysis of PO activity in males for hypothesis 2.

Source	DF Num	DF Den	F	P<
Male				
Treatment	2	265	1.25	0.2891
Population (Trt)	9	265	0.539	0.8344
Rep *	2	9	0.541	0.6
Plate [Rep] *	9	265	6.794	0.0001

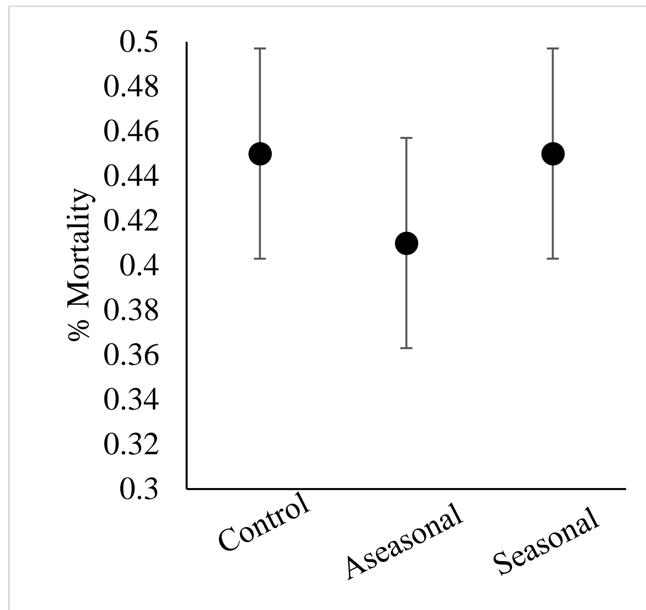

Figure (Appendix 2) - Figure showing results of percent mortality assessment from hypothesis 2.

Table (Appendix 2) - Table showing statistical analysis for percent mortality assessment for hypothesis 2.

Source	**Nparm**	**x2**	**P<**
% Mortality			
Both Sexes			
Treatment	2	2.22	0.3295
Population (Trt)	9	4.61	0.8669
Sex	1	5.78	0.0162

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