

Geographic Variation In Post-mating Immune Gene Expression In *Drosophila Melanogaster*

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GEOGRAPHIC VARIATION IN POST-MATING IMMUNE GENE EXPRESSION IN
DROSOPHILA MELANOGASTER

by

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B.S. Long Island University 2006

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biology
in the College of Sciences
at the University of Central Florida
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ABSTRACT

An organism's immune response may vary due to pathogen pressure in its environment, as well as due to interactions with other organisms. These factors, along with geographic rules (i.e. Gloger's rule) may influence the geographic distribution of the immune response within populations of a species. Here we use real-time quantitative PCR to measure the immune gene expression in six populations collected along the eastern U.S. of *Drosophila melanogaster* after mating. Antimicrobial genes did not show significant differences in expression due to location, whereas we did observe differences in anti-fungal and pro-phenoloxidase (anti-macromolecule) related genes. These differences in anti-macromolecule resistance are correlated with the latitude of the population opposite of which we would expect by Gloger's rule. We also determined that males and females from different populations tended to drive the differences we detected. Taken together, these results suggest that geographic factors influence genes involved in fungal and macro-pathogens defense post-mating.

I dedicate this thesis to the two people who have held the most importance in my life. My grandmother Virginia, who is the strongest woman I've ever met and my mother Linda, who would have been extremely proud.

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I would like to acknowledge several people for their help and support through this process. First, I would like to thank my committee members Dr. Ken Fedorka, Dr. Jane Waterman, and Dr. Eric Hoffman. Their insights and guidance have been invaluable.

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INTRODUCTION

Functional differences between individuals that are related to the survival and reproduction are subject to natural selection and may be influenced by the environment, which can lead to local adaptation and genetic differentiation. Many environmental factors can act as selective pressures, which result in clinal variation of these differences at the continental scale. Pigmentation is an important trait involved in processes such as sexual selection, UV protection, thermoregulation, and immunity. 'Gloger's rule' is based on the observation that organisms with darker pigmentation generally tend to be found in more warm and humid environments, and there is selection pressure along a latitude gradient that maintains variation in pigmentation. Many studies have supported Gloger's rule [1-7], however many have also found contradictory or inconsistent patterns[8-11]. Typically, studies investigating geographic variation in pigmentation have been concentrated in morphological (e.g. body size) and life-history traits (e.g. growth and reproduction). Although much has been learned from observing these traits, it has been more difficult to observe variation in physiological traits. Recent advances in molecular techniques now allow researchers to easily measure changes in organism physiology and determine whether they are also correlated with environmental gradients. One important trait is immune function, considering its critical role in survival (i.e. pathogen defense), reproduction and life history evolution [12-14]. Lines of *Drosophila falleni* that were selected for low pigmentation were more susceptible to a nematode infection than wild type flies [15]. If disease can also influence pigmentation patterns, it is not clear whether immunity would be a trait that is geographically consistent with Gloger's rule.

Several studies have examined how immune function varies geographically, especially with regard to latitude. Considering that latitude tends to be strongly associated with species diversity [16], infection risk may be greater in the tropics than at temperate latitudes. In the white-crowned sparrow (*Zonotrichia leucophrys*) there is a general trend towards increased pathogen risk as latitude decreases in the northern hemisphere [17]; however, it is unclear if this trend is due to life history trade-offs or a result of increased diversity of pathogens at lower latitudes. In a study of pathogens in *Drosophila melanogaster*, host populations were found to differ in their bacterial communities although this variation did not vary with latitude or temperature [18]. Interestingly, a follow-up study showed that, *D. melanogaster* populations that naturally encountered a greater variety of pathogen species were better able to resist infection [19]. Thus, populations of *D. melanogaster* appear to be adapted to resist local pathogen diversity, although this adaptation appears to be independent of latitude. However, it is unclear if this pathogen risk is coming from an environmental source or from interactions with other individuals. In the common house sparrow (*Passer domesticus*), Neotropical birds had relatively stable immune function year round, whereas north-temperate birds showed stronger immune function during nonbreeding season suggesting that the act of mating can strongly influence immunity [20].

In addition to the pathogen risks associated with everyday life, many organisms exhibit a substantial immune response after mating, which could be a defense of sexually transmitted diseases (STDs). Previous work supports the hypothesis that an organism's geography plays a large role in the rate of STD infection. For example, the order Coleoptera exhibits the most STDs of any order of insect. Across coleopteran species, there appears to be a greater degree of

infection in tropical areas, suggesting a latitudinal spatial structure in STD distribution.

Furthermore, most insect STDs are transmitted both sexually and horizontally [21]. This has been experimentally tested in *D. melanogaster*, where both courtship and mating were shown to increase the transmission of the gram-negative bacteria *Serratia mercescens* [22]. For instance, in the fruit fly, *D. melanogaster*, both males and females exhibit a dramatic but transient increase in the production of antimicrobial peptides (Amps) that target bacterial pathogens in the hours directly following mating [23, 24]. However the most common STDs in *D. melanogaster* are fungus and macro-parasites, and no bacterial pathogens have been reported [21].

Here we investigated geographic variation for post-mating immunity in *Drosophila melanogaster* collected along the eastern United States and discuss the potential implications of this adaptive genetic variation. Previous work in *D. melanogaster* suggests the species is largely panmictic across the eastern U.S [25]. However, local adaptation is possible as long as selection is strong enough to overcome the effects of migration, particularly in physiological traits. For instance, local adaptation due to latitudinal pressures has been identified in a large number of traits despite high gene flow estimates[26]. Furthermore, it appears that both sexes emphasize different parts of the immune response, with males tending to emphasize gram negative pathways and females tending to emphasize gram positive and fungal pathways [23, 24, 27]. Thus, any differences we see between populations may be due to variation in immunity in either sex. In this study, we examine (1) geographic variation in the post mating immune response by looking at immune gene expression in females, (2) the association between immune response and latitude to determine if environmental factors could be important in the spatial distribution of this physiological trait, (3) whether the sexes can differentially impact this variation, (4) whether

males from different populations influence female immune response differently, and (5) if those difference may be influenced by the geographic distance of its mate (i.e. isolation by distance).

METHODS

Background

The innate immune system is the primary system responsible for invertebrate immunity and has been highly conserved across most taxa. Invertebrate innate immunity consists of two main components: 1) the humoral component and 2) the cell-mediated component (Table 1). The humoral component is responsible for producing antimicrobial peptides (Amps) as well as pro-phenoloxidase, which aids in cell mediated responses. Amp pathways include *Toll* and *Imd*. The *Toll* pathway is primarily involved in defense against gram-positive bacterial pathogens; the *Imd* pathway is involved in defending against gram-negative pathogens. The cell-mediated component involves both phagocytosis of pathogenic invaders and the encapsulation response. The encapsulation response involves the aggregation of hemocytes around a macromolecule, where hemolymph and cell bound pro-phenoloxidase lead to the melanization of the intruder, such as a parasitic wasp egg. The insect immune system is highly specialized, while it remains able to address a wide variety of pathogens [28].

Study Populations

To examine geographic variation in the female immune response to mating, we sampled six populations of *D. melanogaster* at varying latitudes. Gravid females were collected near Orlando, Florida in 2008 (34 founding females), Columbia, South Carolina in 2008 (27 founding females), Macon, Georgia in 2005 (12 founding females), Long Island, New York in 2006 (22 founding females), Belmont, Vermont in 2007 (30 founding females), and Sudbury, Ontario in 2007 (30 founding females) (Figure 1). These populations were maintained on a standard

cornmeal-yeast medium as isofemale lines, until all progeny from those lines were combined into a representative outbred population at each location no earlier than 2 generations before the experiment. Isofemale stocks were maintained in 30ml vials, whereas outbred stocks were maintained in 30cm³ population cages.

Mating and immune assays

All experimental flies were separated by sex upon adult eclosion and housed in individual food vials until they were 5-7 days old. To examine the post mating immune response, flies were randomly mated within (homotypic) and across (heterotypic) populations by combining male and female vials without the use of anesthetic; creating one mating pair per vial. These pairs remained together for up to 2 hours and were checked every 10 minutes. The time at which mating was observed was recorded, and any unsuccessful matings were discarded (<4%). Successfully mated females were lightly anesthetized (CO₂) and separated into groups of 7 that mated within a half an hour of each other. Seven females from each treatment should capture adequate genetic variation in response. Six hours after mating, females were transferred into Trizol (Invitrogen). We chose to study the six-hour time point, as it has shown the greatest response in female expression of several immune genes [27, 29].

To assay the post mating immune response, we estimated transcript levels for several key immune genes involved in both the humoral and cell mediated response. The humoral antimicrobial genes chosen were *attacinA* and *cecropinA1* from the *Toll* pathway. From the *Imd* pathway, *drosomycin1* is an anti-fungal peptide that is constitutively expressed in the reproductive tract of female *D. melanogaster* [30]. The final antimicrobial gene chosen was

defensin, which is controlled by both *Toll* and *Imd* pathways. From the cell mediated immune response we chose regulatory genes of the encapsulation response, *hemese* and *pro-phenoloxidase AE* (Table 1) [28].

We used a chloroform/isopropanol protocol to extract RNA and reverse transcribed the RNA into cDNA with the Invitrogen two-step RT-PCR kit. We measured relative gene expression levels with quantitative PCR (Biorad) using SYBR Green Supermix. The constitutively expressed gene *Actin-5* was used as a reference to calculate the relative quantity of gene transcript present. This was done by comparing the change in the PCR cycle at which a particular gene was amplified relative to the cycle at which *Actin-5* was amplified at a certain threshold (Cycle threshold (Ct)). The change in cycle threshold (dCt) measures the change in cycle threshold in target gene relative to the reference gene.

$$Ct = Ct(\text{reference}) - Ct(\text{gene of interest}) \quad (\text{Equation 1})$$

Data analysis

Means of 3 replicates of cycle thresholds from each sample were calculated. The data were compiled in Microsoft Excel 2007. Dixon tests for outliers were performed ($\alpha=0.05$), and outliers were winsorized (<2%). The data did not meet expectations for normality, thus nonparametric statistical methods were applied, using sequential Dunn-Sidak for multiple testing correction [31]. All analyses were performed in JMP 8 (SAS Institute Inc., Cary, NC). Nonparametric Kruskal-Wallis tests were used to detect overall differences in mean gene expression between populations, and Mann Whitney U-tests were performed between pairs of populations. Nonparametric Spearman correlations were used to detect associations between

mean dCt and latitude of population location and geographic distance between populations. A nonparametric 2-way ANOVA using Scheirer-Hare Ray extension [31] of Kruskal Wallis tests were used to determine the effects of male and female populations. Finally, Kruskal-Wallis tests were performed between homotypic and heterotypic populations.

RESULTS

To determine if geographic variation exists for the female immune response to mating, we examined the differences in mean dCt for all immune genes across all populations. The genes *attacinA*, *cecropinA1*, and *defensin* did not show significant overall differences in mean expression between populations using Kruskal-Wallis tests with sequential Dunn-Sidak correction [31]. Significant differences among populations were found in *drosomycin1* ($H_{dmy1} = 16.7779$, 5 d.f., $P=0.0049$; Figure 2), *hemese* ($H_{hem} = 24.8123$, 5 d.f., $P=0.0002$; Figure 3), and *pro-phenoloxidase AE* ($H_{proAE} = 30.3820$, 5 d.f., $P<0.0001$; Figure 4). To further examine differences between each population within a given gene, we performed Mann-Whitney *U*-tests.

To determine if there was a latitudinal association across homotypic populations with female immune gene expression we correlated the mean female immune response (mean dCt) with latitude using Spearman correlations with a sequential Dunn-Sidak correction. There were no significant correlations using 6 populations, considering that the Georgia population exhibited the highest variation for most immune genes and was established with the fewest number of wild females, it may be that the Georgia population had a large and erroneous influence on our results. Thus, we removed the Georgia population and reanalyzed the data. We found *hemese* (Spearman's $\rho_{hem} = 1.0000$, $P = 0.0000$; Figure 5) and *pro-phenoloxidase AE* (Spearman's $\rho_{proAE} = 1.0000$, $P = 0.0000$; Figure 6) were significantly positively correlated with latitude. However, there was a small sample size ($n=5$ without Georgia, $n=6$ with Georgia) for each test.

To identify whether the female immune response appeared to be driven primarily by males from a particular geographic location, or whether females from a specific location had a

large degree of post-mating immune response, we performed the nonparametric two-way ANOVA (Scheirer-Ray Hare extension of the Kruskal-Wallis test; [31]). We tested for effects of location of each sex and the interaction between the geographic locations of each of the mates using a sequential Dunn-Sidak correction. We removed the Georgia populations from the analysis (Table 2), and found a significant effect of the male population location in the female post-mating gene expression of *attacin A* and *drosomycin I*. Regardless of female location, expression of *attacin A* was almost always higher when mated to males from the Ontario population (Figure 7) and expression of *drosomycin I* was almost always higher when mated to males from the South Carolina population (Figure 8). A significant effect of the location of the female population was also detected in the post-mating expression of *hemese* and *prophenoloxidase AE*. For *hemese*, females from the New York population usually showed lower expression and females from Ontario usually had higher expression regardless of their mate's location (Figure 9). The expression of *prophenoloxidase AE* was always lower for females from Florida and usually higher for females from Ontario, regardless of the male's geographic location (Figure 10). When we performed this analysis with the Georgia population included (Table 3), we found the same significant effects of the previous analysis, with females from Georgia always showing higher post-mating gene expression in *hemese* regardless of male populations (Figure 11). Significant effects of the male population location in the female post-mating expression of *cecropin A1* and *prophenoloxidase AE* were also detected, however the population specific patterns were unclear (Figures 12-13).

To determine if there was an influence of heterotypic males on female immune gene expression after mating, we performed Mann Whitney *U*-tests with sequential Dunn-Sidak

correction. Previous results have suggested that foreign males elicit higher immune responses than homotypic males (Pinzone unpub. results). We found a marginal difference in *defensin* ($U_{def} = 2.8829$, 1 d.f., $P = 0.0895$; Figure 14), where heterotypic matings elicited higher female immune gene expression than homotypic matings.

To identify whether isolation by distance geographic patterns exist, we performed Spearman correlations between mean dCt and the geographic distance (in Km) between males and females of heterotypic matings. No significant associations between female immune gene expression and distance were found, even after removing the Georgia population.

DISCUSSION

In this study, we examined geographic variation of post-mating immunity in female *Drosophila melanogaster*. Specifically, we found that in several of the genes assayed that geographic variation does exist in the post mating immune response, there is an association between the post mating immune response and the population's latitude, and males and females can differentially influence this geographic variation. We did not detect a significant effect of mating type, or an isolation by distance pattern.

We found that females from different populations differed in post-mating gene regulation for several genes. We did not see any differences in genes that are related to the *Toll* pathway of the humoral immune system. Interestingly, the most common STDs in *Drosophila* are fungus and macro-parasites, and no evidence of bacterial pathogens has been identified [21]. Accordingly, we find no difference for the antibacterial peptides *attacinA*, *cecropinA1* and *defensin* between populations, but find differences in the antifungal gene *drosomycin1* and the pro-phenoloxidase associated genes *hemese* and *pro-phenoloxidase AE*.

When looking across latitude, we found data points from the Georgia population highly variable, possibly due to the fact that it was started with the fewest isofemale lines and were maintained in the laboratory for the longest time. When we removed Georgia from the analysis we found that all immune genes that exhibited differences between populations also exhibited an association with latitude (non-*Toll* genes). Counter to the Gloger's rule, the pro-phenoloxidase genes *hemese* and *pro-phenoloxidase AE* exhibit a greater post-mating gene expression in the northern latitudes. Work by McKean and colleagues have identified a similar pattern in baseline

immune function along a latitudinal cline (McKean, personal communication). The outcome of the melanization pathway may indeed be greater, depending on how it is regulated by *hemese* and *prophenoloxidase AE*. Regardless, these trends do suggest that there is local adaptation for the non-*Toll* genes. The panmictic nature of this species along the eastern seaboard suggests that these observed geographic differences are not likely due to genetic drift, and therefore these differences may have an adaptive basis.

We examined population specific responses to determine if the female immune response appears to be driven primarily by males from a particular geographic location, or by females from a specific location with a large degree of post-mating immune response. When removing Georgia from the analysis, males from the Ontario population elicit higher values of *attacin A* and males from South Carolina elicit higher levels of *drosomycin 1* from females of any population. Regardless of the male population, females from the New York population exhibit lower expression levels of *hemese* and females from Ontario higher levels of *hemese*. For *prophenoloxidase AE*, females from Florida exhibit lower expression levels and females from Ontario higher levels. When we kept Georgia populations in the analysis, we received the same results as well as found that females from Georgia exhibit higher expression levels *hemese* regardless of male population. Male populations showed significant effects on *cecropin A1* and *prophenoloxidase AE*, however with no clear pattern. Population specific life history traits such as changes in the timing of diapause may play a large role in these patterns.

We compared differences in post-mating immune gene expression between matings from the same location (homotypic) versus matings of flies from different locations (heterotypic) and

did not see statistically significant differences. We did observe a marginally significant difference in *defensin*, where the heterotypic immune response was higher than the homotypic response. Nor did we find evidence for isolation by distance between males and females from these populations.

Future work to test different paralogs of the antimicrobial peptide genes assayed here may show a different geographic pattern and should be investigated. Other regulatory genes in the encapsulation pathway can also be investigated, however this information will be most valuable once more is known how the pathway work and the relative role of each of its regulators. Another useful piece of information to collect is the inducibility of each of these genes in the different populations of the virgins versus the mated individuals. The female immune gene inducibility is known to be different depending on the mate [24]. Finally, testing the survival of infection of individuals from these populations may allow us to determine how immune gene expression relates to actual immune function, and determine whether post-translational processes may be important to the function of these proteins [29].

APPENDIX: FIGURES AND TABLES

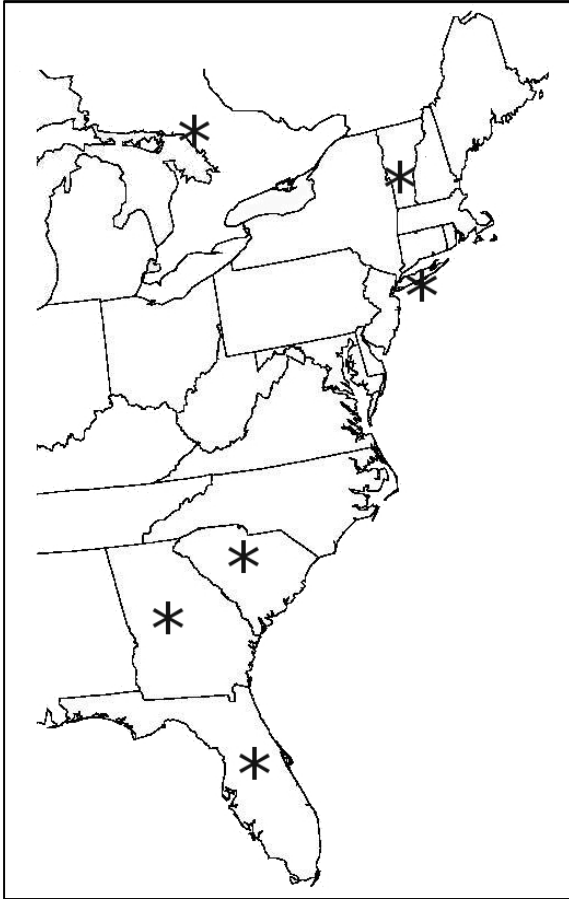


Figure 1: Locations of collections of samples of *D. melanogaster*. From North to South: Sudbury Ontario, Belmont Vermont, Old Brookville New York, Columbia South Carolina, Macon Georgia, and Orlando Florida.

Table 1: Paralogs of immune genes assayed including the part of the immune response, pathway, function, gene family and gene ID. * = also regulated by *Toll* pathway

Immune pathway	Functional group	Gene family	Paralog	Gene ID
Humoral	targets gram negative bacteria (<i>Imd</i> pathway)	<i>defensins</i> * <i>drosomycins</i>	<i>def</i> <i>dmy1</i>	CG1385 CG10810
	targets gram positive bacteria (<i>Toll</i> pathway)	<i>attacins</i> <i>cecropins</i>	<i>attA</i> <i>crpA1</i>	CG10146 CG1365
Cell mediated	regulation of encapsulation	<i>hemese</i> <i>serine</i> <i>proteases</i>	<i>hem</i> <i>proAE</i>	CG31770 CG9733

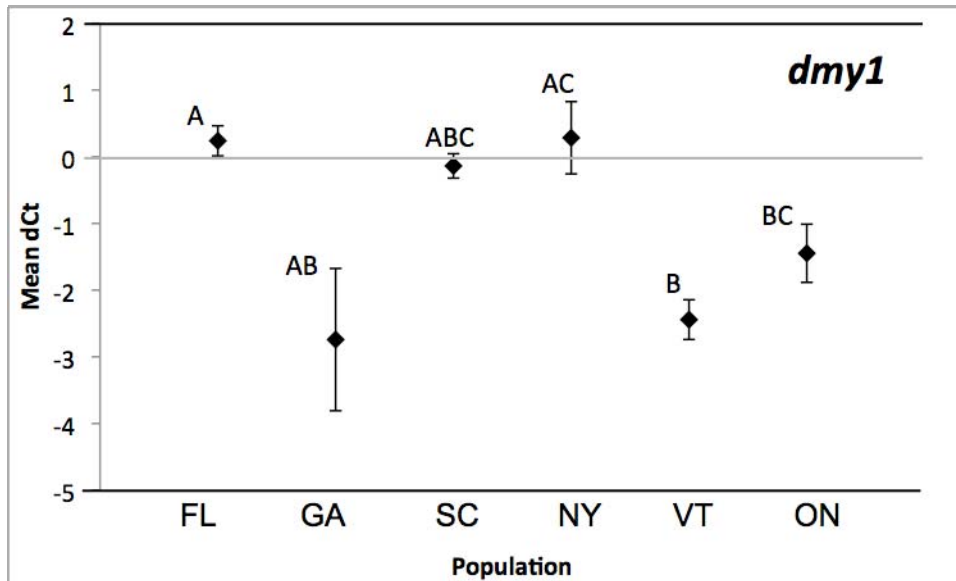


Figure 2: Mean and standard error of female gene expression after mating within each population for *drosomycin1*. Kruskal Wallis test detected significant differences among populations and Mann Whitney *U*-tests detected significant differences between populations shown by different letters.

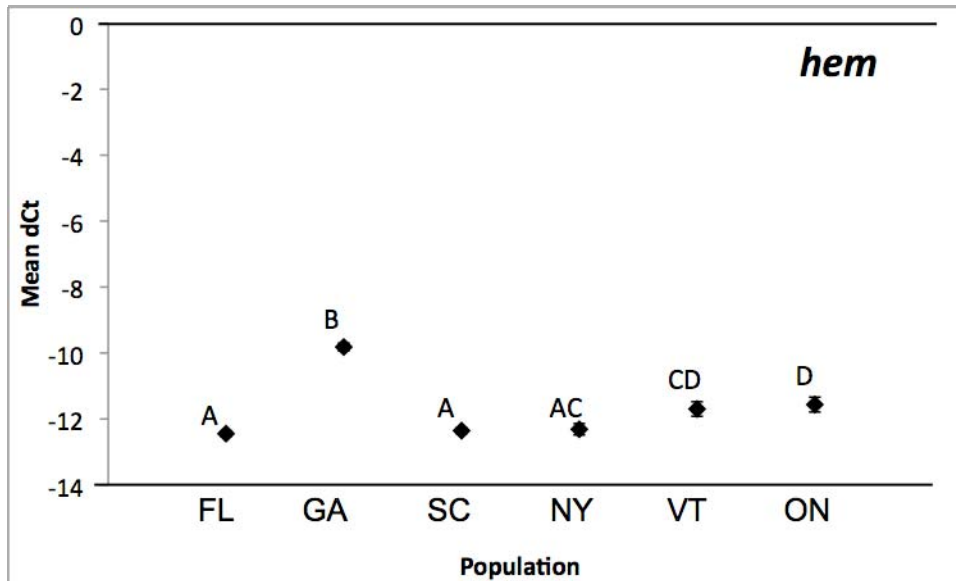


Figure 3: Mean and standard error of female gene expression after mating within each population for *hemese*. Kruskal Wallis test detected significant differences among populations and Mann Whitney *U*-tests detected significant differences between populations shown by different letters.

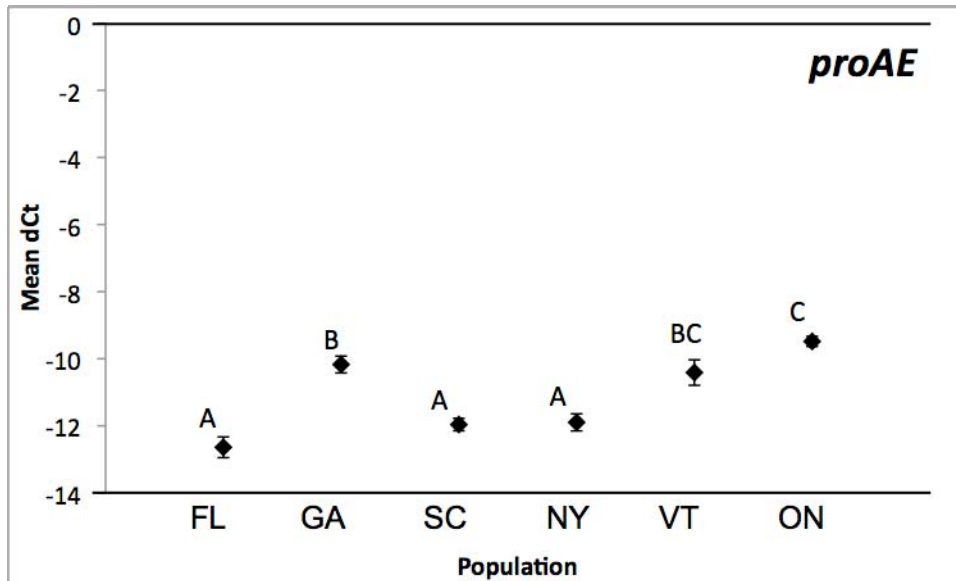


Figure 4: Mean and standard error of female gene expression after mating within each population for *pro-phenoloxidase AE*. Kruskal Wallis test detected significant differences among populations and Mann Whitney *U*-tests detected significant differences between populations shown by different letters.

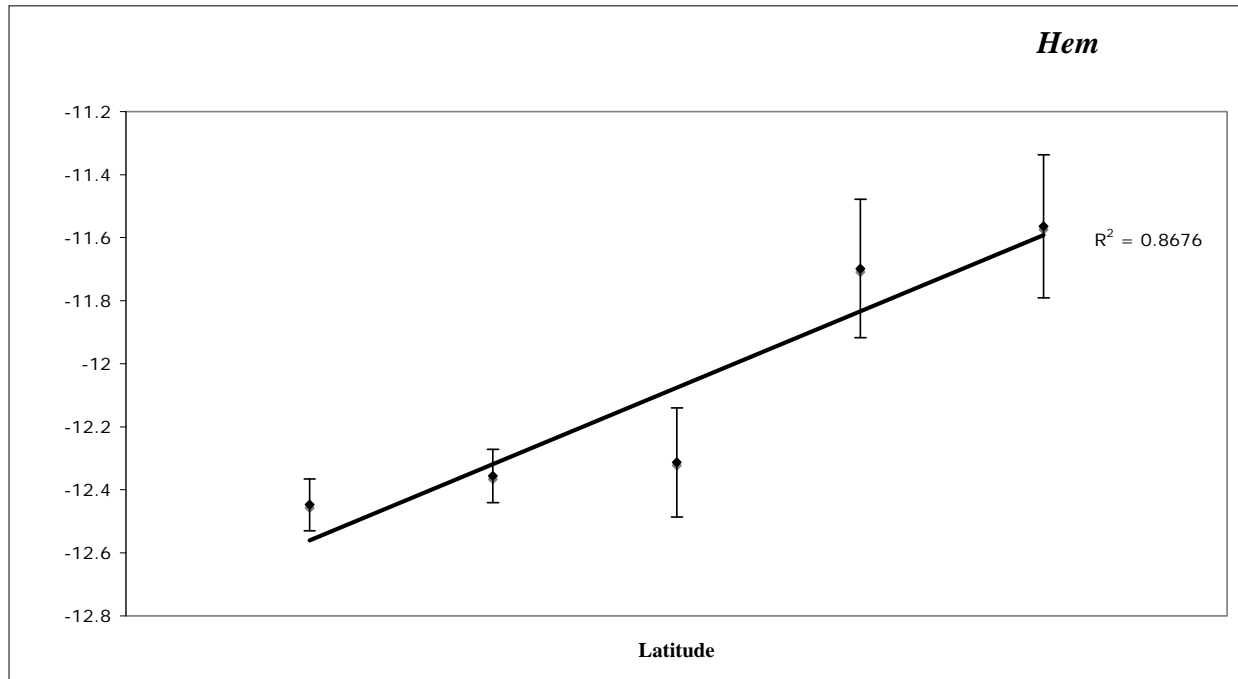


Figure 5: Mean gene expression and standard error values of *hemese* in females after mating plotted against the latitude at which they were collected, Georgia females were removed from the analysis. A significant positive association was detected.

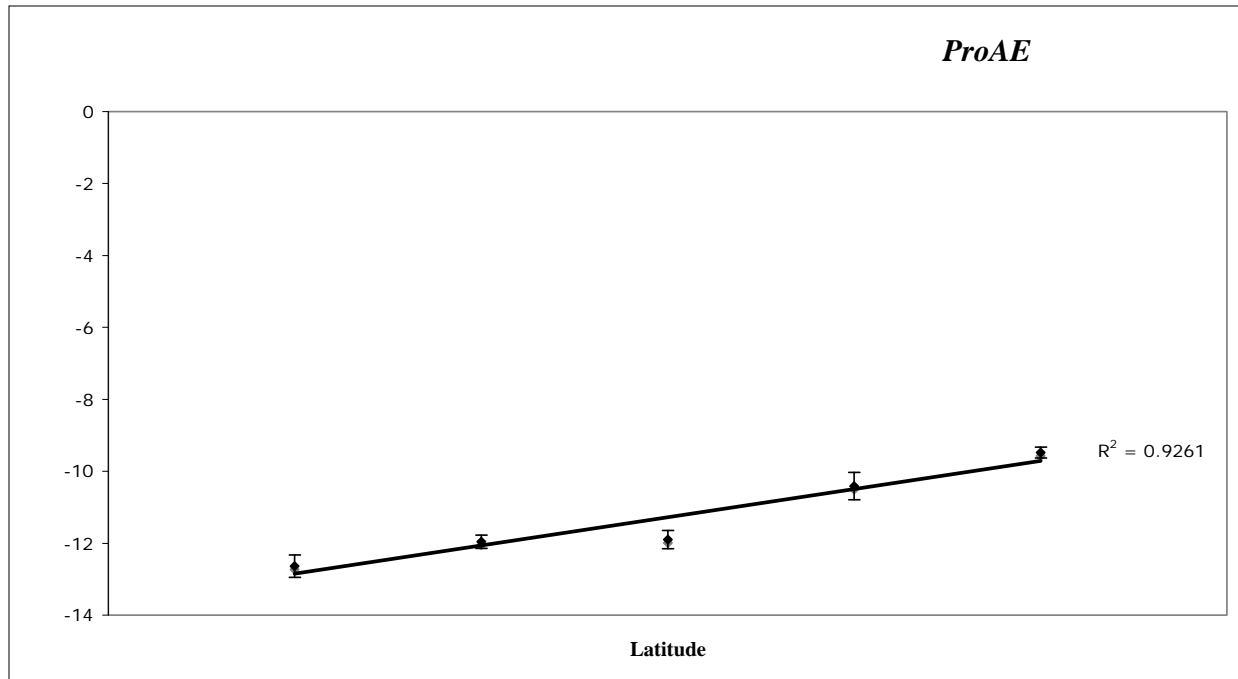


Figure 6: Mean gene expression and standard error values of *prophenoloxidase AE* in females after mating plotted against the latitude at which they were collected, Georgia females were removed from the analysis. A significant positive association was detected.

Table 2: Effect of male and female population and their interaction on female immune gene expression after mating. Nonparametric two-way ANOVAs (Scheirer-Ray Hare extension of the Kruskal-Wallis test) of six immune genes. * = statistical significance at $\alpha = 0.05$ with sequential Dunn-Sidak correction.

Gene	Source of variation	d.f.	SS	<i>H</i>	<i>P</i>	
<i>attA</i>	Male Pop	5	3416.87	11.62	0.0404	*
	Female Pop	5	1787.03	6.08	0.2989	
	Male X Female Pop	25	5090.51	17.31	0.8703	
<i>crpA1</i>	Male Pop	5	11498.72	12.41	0.0295	*
	Female Pop	5	6393.89	6.90	0.2281	
	Male X Female Pop	25	14538.28	15.69	0.9237	
<i>def</i>	Male Pop	5	357.48	0.91	0.9692	
	Female Pop	5	2115.15	5.41	0.3683	
	Male X Female Pop	25	11221.56	28.68	0.2774	
<i>dsm1</i>	Male Pop	5	1893.58	11.95	0.0355	*
	Female Pop	5	924.92	5.84	0.3224	
	Male X Female Pop	25	2728.25	17.22	0.8738	
<i>hem</i>	Male Pop	5	1444.89	4.46	0.4851	
	Female Pop	5	6483.22	20.01	0.0012	*
	Male X Female Pop	25	3409.44	10.53	0.9949	
<i>proAE</i>	Male Pop	5	6786.14	12.28	0.0312	*
	Female Pop	5	8055.97	14.57	0.0123	*
	Male X Female Pop	25	4506.94	8.15	0.9994	

Table 3: Effect of male and female population and their interaction on female immune gene expression after mating, Georgia populations have been removed. Nonparametric two-way ANOVAs (Scheirer-Ray Hare extension of the Kruskal-Wallis test) of six immune genes. * = statistical significance at $\alpha = 0.05$. with sequential Dunn-Sidak correction.

Gene	Source of variation	d.f.	SS	H	P	
<i>attA</i>	Male Pop	4	1267.76	9.55	0.0486	*
	Female Pop	4	504.96	3.80	0.4330	
	Male X Female Pop	16	1412.64	10.64	0.8309	
<i>crpA1</i>	Male Pop	4	3268.70	8.61	0.0716	
	Female Pop	4	1760.10	4.64	0.3267	
	Male X Female Pop	16	4082.70	10.75	0.8244	
<i>def</i>	Male Pop	4	185.04	0.83	0.9342	
	Female Pop	4	837.84	3.76	0.4393	
	Male X Female Pop	16	4323.73	19.41	0.2480	
<i>dsm1</i>	Male Pop	4	703.36	9.85	0.0430	*
	Female Pop	4	202.96	2.84	0.5846	
	Male X Female Pop	16	807.84	11.31	0.7899	
<i>hem</i>	Male Pop	4	993.04	7.51	0.1111	
	Female Pop	4	1321.84	10.00	0.0404	*
	Male X Female Pop	16	857.36	6.49	0.9819	
<i>proAE</i>	Male Pop	4	1888.54	6.68	0.1537	
	Female Pop	4	3666.54	12.97	0.0114	*
	Male X Female Pop	16	1228.66	4.35	0.9981	

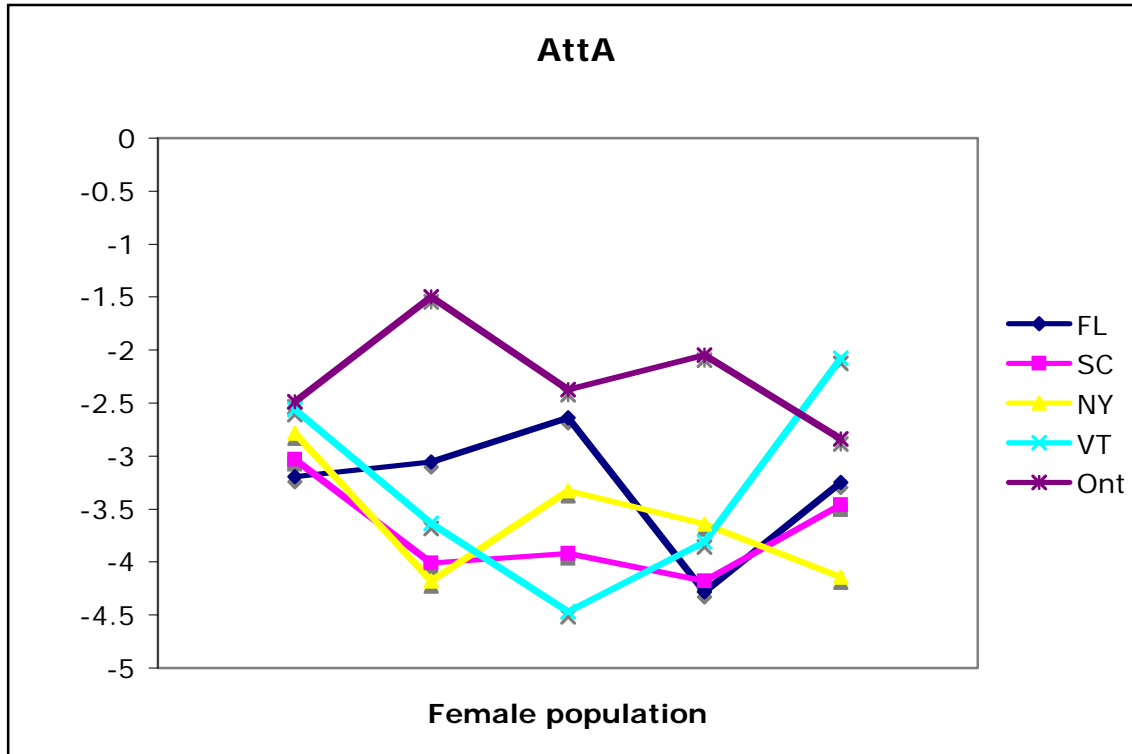


Figure 7: Mean female post-mating gene expression of *attacin A* by each female population location (x axis) and male population location (see legend), Georgia removed from analysis.

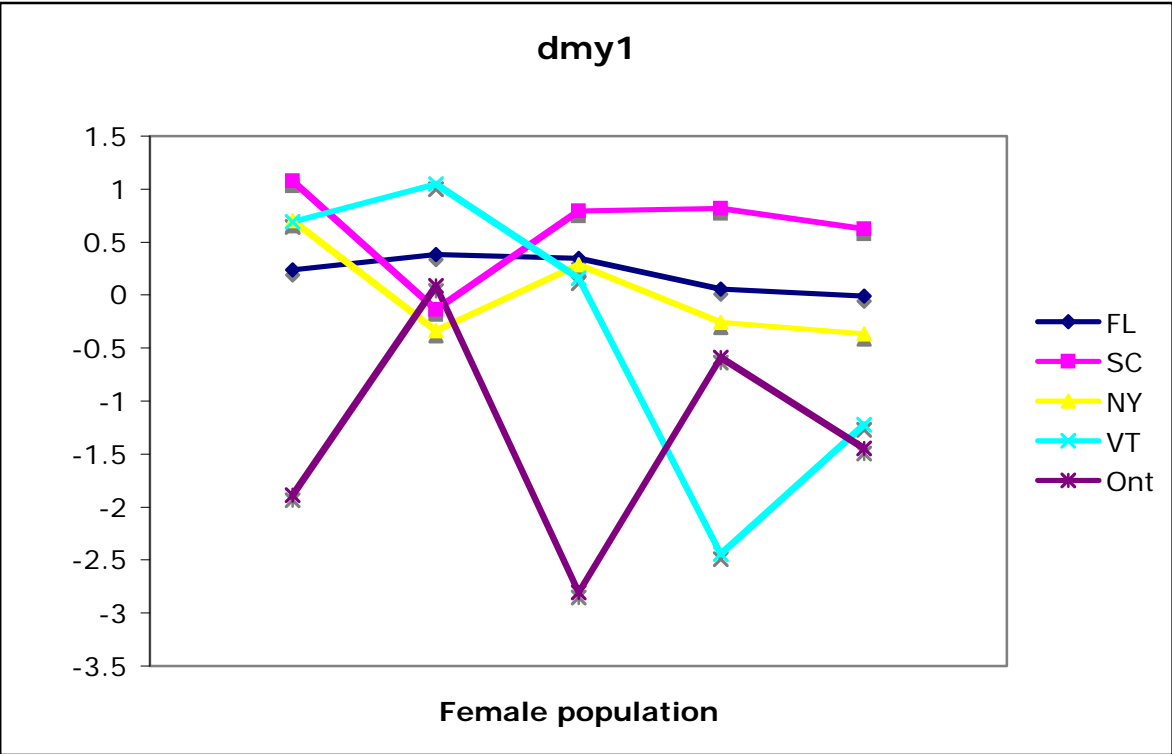


Figure 8: Mean female post-mating gene expression of *drosomycin1* by each female population location (x axis) and male population location (see legend), Georgia removed from the analysis.

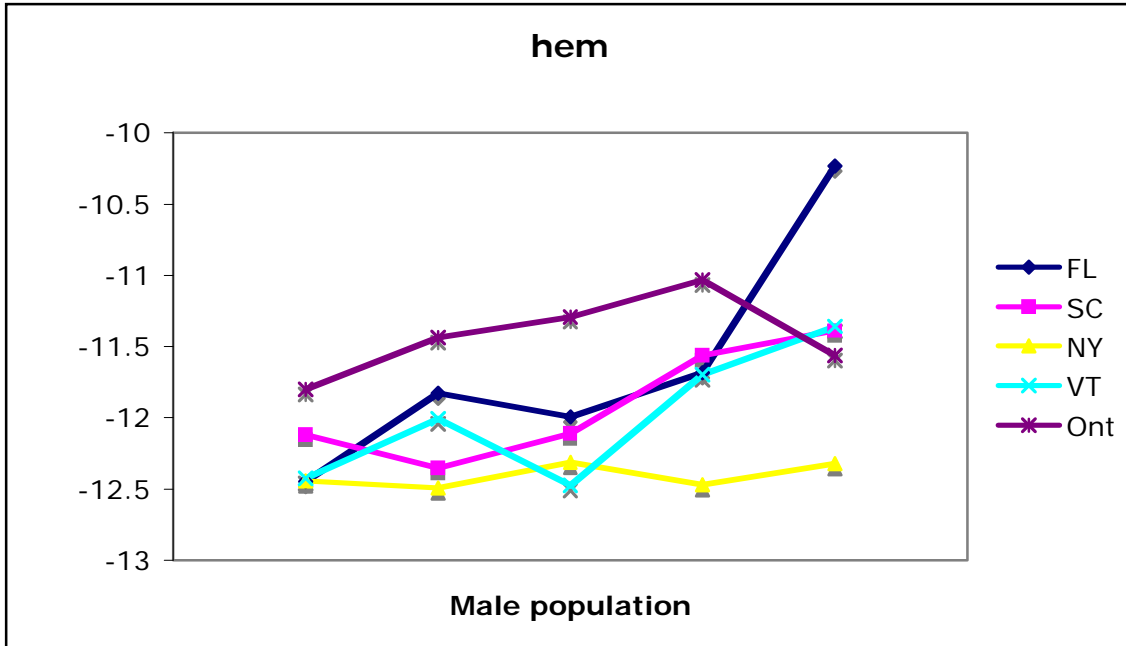


Figure 9: Mean female post-mating gene expression of *hemese* by each male population location (x axis) and female population location (see legend), Georgia removed from the analysis.

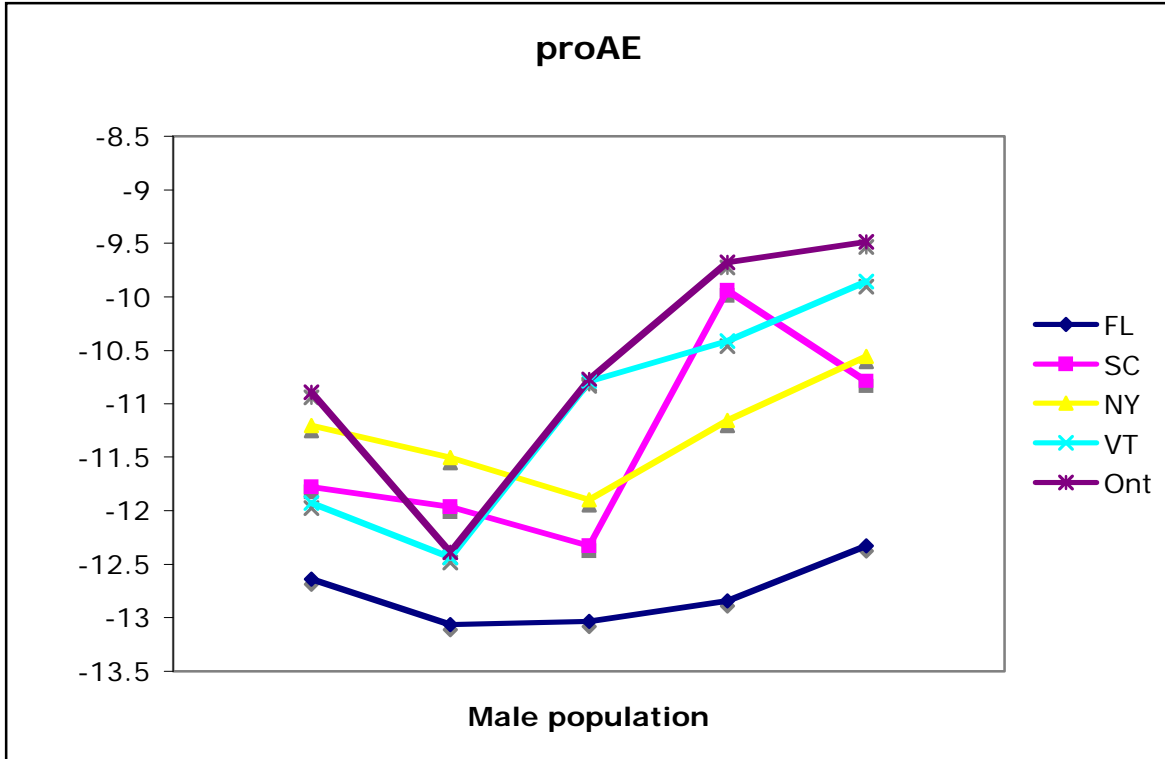


Figure 10: Mean female post-mating gene expression of *prophenoloxidase AE* by each male population location (x axis) and female population location (see legend), Georgia removed from the analysis.



Figure 11: Mean female post-mating gene expression of *hemese* by each male population location (x axis) and female population location (see legend).

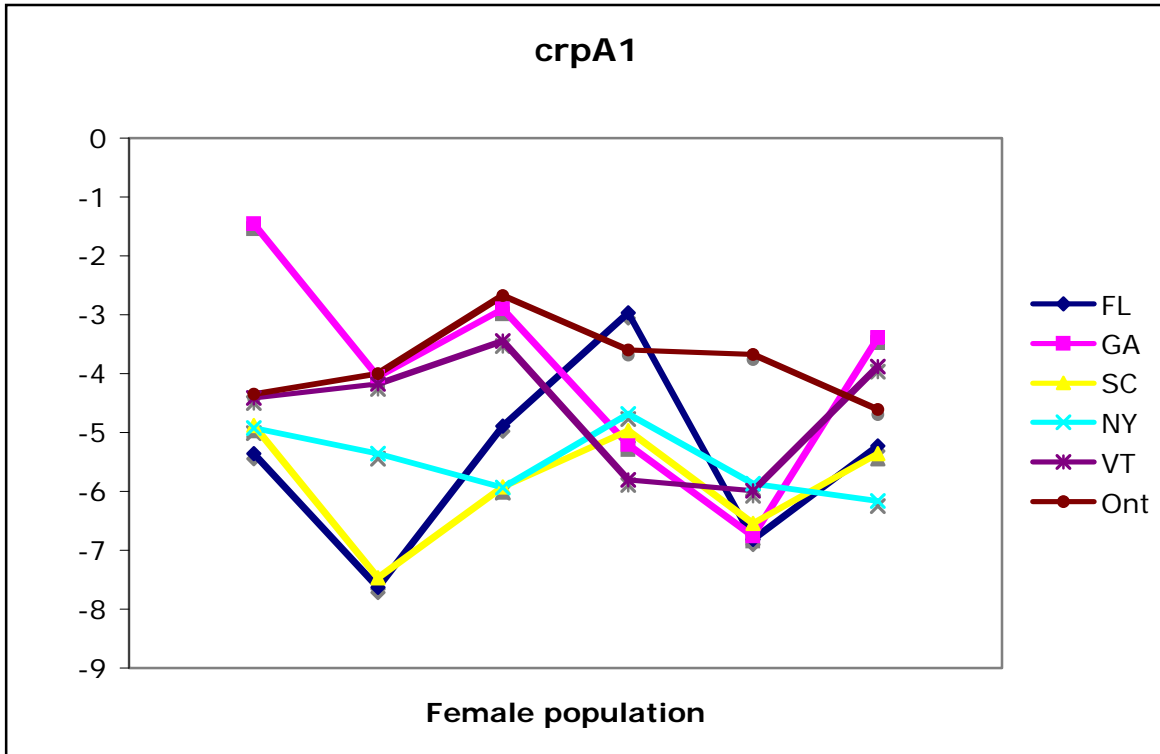


Figure 12: Mean female post-mating gene expression of *cecropin A1* by each female population location (x axis) and male population location (see legend).



Figure 13: Mean female post-mating gene expression of *prophenoloxidase AE* by each female population location (x axis) and male population location (see legend).

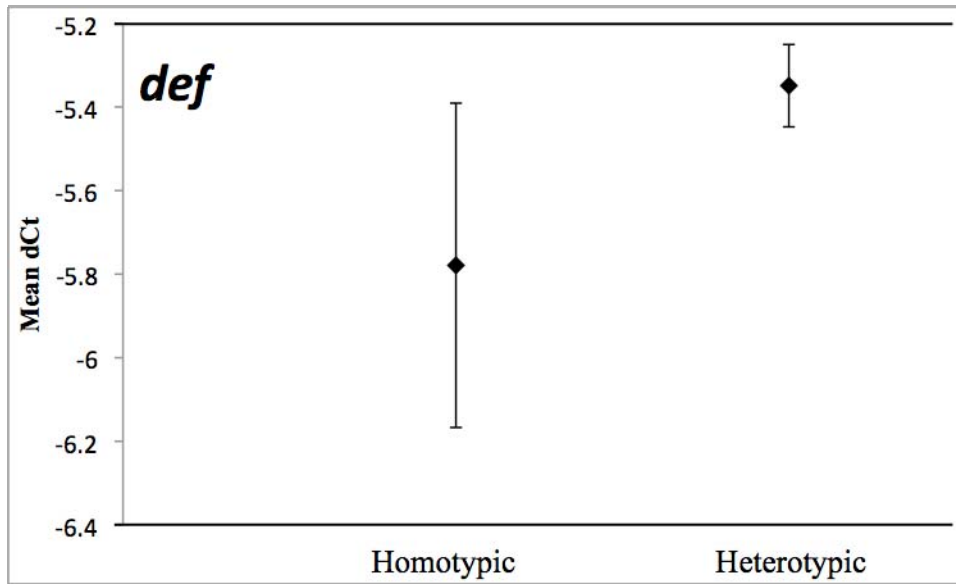


Figure 14: Mean and standard error of female gene expression of *defensin* after mating with males from the same population versus males from different populations. Mann Whitney *U*-test detected a marginal difference.

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