SYMPATRIC, ALLOCHRONIC POPULATIONS OF THE PINE WHITE BUTTERFLY *(NEOPHASIA MENAPIA)* ARE MORPHOLOGICALLY AND GENETICALLY DIFFERENTIATIED

THESIS

Presented to the Graduate Council of
Texas State University–San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

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San Marcos, Texas
December 2012

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ACKNOWLEDGEMENTS

I would like to thank Arthur Shapiro (UC Davis), who discovered the two sites with sympatric populations. Thank you to Jeff Oliver, Katy Prudic, Erik Runquist, Chris Nice and Arthur Shapiro for collection of specimens. Thank you to Chris Nice, as the chair of my committee, Jim Ott and Noland Martin as committee members. I would also like to thank the members of the Nice lab group and the EEB discussion group at Texas State University-San Marcos.

This manuscript was submitted on July 20th 2012.

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ABSTRACT

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SUPERVISING PROFESSOR: CHRIS NICE

Allochronic isolation remains an understudied, and potentially under-appreciated, mechanism of reproductive isolation. Allochrony has been detected in two sympatric populations of the pine white butterfly (*Neophasia menapia*); a typically univoltine species found throughout Western North America. At two sites in California there are early and late flights. Differences in flight time are accompanied by differences in wing shape and pigmentation. A combination of morphological analysis and population genomics was used to assess population differentiation at both sites with sympatric populations. Differences in wing shape and pigmentation between early and late flights were quantified. Pigmentation was significantly different at both sympatric sites, while shape was significantly different at just one of the sites. 20,737 SNP's, obtained using next generation sequencing, were used to calculate pairwise F_{ST}'s. F_{ST} between early and late flights was significantly different from zero at both sympatric sites. The hypothesis of a double invasion from Sierra Nevada sites to Coastal Range sites, leading to the two flights at sympatric sites, was rejected. Allochrony is likely to have arisen in sympatry, from within the Coastal Range.

CHAPTER I

THESIS

INTRODUCTION

Allochronic isolation arises when differences in phenology lead to reproductive isolation (Alexander & Bigelow 1960, Coyne & Orr 2004). Relative to other mechanisms of reproductive isolation, it is infrequently studied and possibly under-appreciated (Coyne & Orr 2004). The potential for temporal shifts in life history to limit gene flow is best studied in sympatry in order to eliminate the confounding effects of geographic isolation (Abbott & Withgott 2004, Friesen *et al.* 2007). In recent years there have been several examples indicating that allochrony is a potentially important reproductively isolating mechanism, but research into the process is still limited to just a handful of studies (Abbot & Withgott 2004, Friesen *et al.* 2007, Yamamoto & Sota 2009, Ording *et al.* 2010, Santos *et al.* 2011a, Santos *et al.* 2011b, Yamamoto & Sota 2012). Here we investigate allochronic isolation in the pine-white butterfly, *Neophasia menapia* (Lepidoptera: Pieridae), at two sympatric sites in California.

Neophasia menapia is a species of butterfly that occurs throughout Western North America (Scott 1992, Guppy & Shepard 2001). The common name of the 'pine white butterfly' refers to larval host choice of species from Pinaceae (Guppy & Shepard 2001). It is a univoltine species; adults emerge in summer, eggs are laid and overwinter until the following spring when they hatch, go through caterpillar and pupal stages, and develop into adults (Fletcher 1905, Elrod 1906, Comstock 1924, Garth 1930, Belvins & Belvins 1944, Brock & Kaufman 2006). In California two locations have been discovered where there are two periods of adult emergence per year, one in *early* summer (July) and one in *late*summer/autumn (September/October) (hereafter referred to as early and late flights respectively). At these sympatric sites differences in emergence time, early or late, appear

to be accompanied by differences in the extent of melanization (black pigmentation) on the wing, as well as differences in wing shape.

Here a combination of morphological analysis and population genetics is used to examine the extent to which these sympatric sites are differentiated and to test hypotheses on the possible origin of these sympatric butterflies. We address three questions: 1). Are the two flights at each of the sympatric sites differentiated from each other, and other nearby *N. menapia* populations, in terms of wing pigmentation and wing shape? 2). Do the two flights at sympatric sites exhibit population genetic differentiation consistent with the hypothesis of sympatric, allochronic isolation? 3). What can we infer about the origin(s) of the sympatric populations? There are two main hypotheses about the origin of sympatric populations. Firstly, double or single invasion hypothesis with colonization occurring from one (or more) of the Sierra Nevada sites or the second hypothesis that sympatric early and late flights arose from within the Coastal Range. The use of a multi-locus data set using markers from across the genome provides fine resolution of genome-wide processes for addressing these latter two questions.

METHODS AND MATERIALS

Sampling and collection

187 butterflies were collected between 1995 and 2002 at several locations across California and Oregon (table 1). 173 *N. menapia* were collected at 5 sites in California (figure 1) and 1 site in Oregon. Two flights, early and late, have been observed at two sites in California – Goat Mountain and Mendocino Pass. At these sympatric sites individuals were collected during both adult flights. 14 *N. terloottii*, the only other species in the genus *Neophasia*, were collected from Arizona as a basis for comparison in the analysis of population structure of *N. menapia*. Samples were kept in -80^{0}C until DNA extraction.

Morphological analysis

Forewings of male *N. menapia* (table 2) were photographed using a digital camera (Sony Cyber-shot HX9V) on a white background with a scale (mm ruler). Photographs were processed using image J software (http://www.rsbweb.nih.gov/ij/). Our sample included more males than females, we therefore decided to only use male wings in order to prevent additional variation due to sex.

Wing Melanization

First the total area of each wing was measured twice using the polygon selection tool. The average of the two measurements was taken as the wing area. Images were changed to type-8 and then made binary. The wand tool was used to select the black (melanized) area of the wing. Any white that was within black areas was selected and total melanization was calculated as black area minus white area. Each measurement was taken twice and the average of the two was used in calculations. A regression of total melanization on wing area was conducted. The residuals of this regression were used in further statistical analysis in order to remove the influence of wing area on total melanization. A one-way ANOVA was conducted on the residuals, then Tukey's HSD post hoc test was used to examine which pairwise comparisons were significantly different.

Wing Shape

Two linear measurements were taken of the wing (again using image J). Measurement 1 (a proxy for wing length) is taken from where the radial vein (or subcoastal vein) meets the wing margin to where the anal vein meets the wing margin and measurement 2 (a proxy for width) is taken from vein M3 from discal cell to where it meets the wing margin (figure 2). Each of the measurements was taken twice and the average of those two measurements was used in all calculations. Measurement 2 was divided by measurement 1 to provide a ratio of

width:length. This ratio provides an approximation of shape. Broadly speaking, a lower ratio would indicate a taller, thinner overall shape while a higher ratio would indicate a shorter, wider wing shape. A one-way ANOVA was conducted on the ratios, then a Tukey's HSD post hoc test to examine which pairwise differences were significant.

Molecular methods

DNA was isolated and purified from each of the sampled butterflies from approximately 0.1grams of thoracic tissue using: (i) Qiagen's DNeasy 250 Blood and Tissue Kit (Qiagen Inc.) in accordance with the manufacturer's protocol or (ii) standard phenol-chloroform protocol (Hillis *et al.* 1996). Genomic library preparation, using a reduced-representation protocol, involves a series of steps to digest DNA, ligate barcoded sequencing adapters to fragments, amplification of fragments, selection of appropriately sized fragments, purification and sequencing on the Illumina sequencing platform (Gompert *et al.* 2010, Gompert *et al.* 2012, Parchman *et al.* 2012). DNA (6µl) was digested using two restriction enzymes, Mse1 and EcoR1. Customized Illumina adaptor sequences and a ten base pair MID (multiplex identifier) barcode were ligated to DNA fragments for each individual. These reactions were carried out in a solution of 0.2825µl molecular grade H_2O, 1.1µl 10 x T4 buffer, 0.55µl 1M NaCl, 0.55µl BSA (1mg/ml), 1µl Mse1 adaptor, 1µl EcoR1 barcoded adaptor, 0.1µl Mse1 enzyme, 0.25µl EcoR1 enzyme, 0.1675µl T4 DNA ligase. Samples were then incubated in a thermocycler at 37^0c for 18 hours. Digested DNA was diluted 3µl in 10µl of molecular grade H_2O. Next, two PCR's were used for each individual using Bio-Rad's iProof^{TM} high- fidelity DNA polymerase. The master mix was made as follows; 9.4µl molecular grade H_2O, 4µl 5x iProof buffer HF, 0.4µl dNTPs, 0.4µl $MgCl_2$, 1.3µl ill primers (mixed illpcr1 and illpcr2), 0.3µl DMSO, 0.2µl iProof polymerase. 17µl of the master mix was added to 3µl of the diluted restriction ligation reaction. Thermocycler settings were as

follows; 98^0C for 30seconds, 30 cycles of: 98^0C for 20 seconds, 60^0C for 30 seconds, 72^0C for 40 seconds and final extension of 72^0C for 10 minutes.

PCR product from all individuals was pooled together then size selected fragments were purified using QIAquick gel extraction (250) kit (Qiagen INC.) as per the manufacturers' protocol. Illumina sequencing platform produces 100bp sequences; therefore fragments between 300-500bp were selected to avoid sequencing short fragments. To ensure that the concentration of DNA is appropriate for sequencing, a nanodrop spectrophometer was used to measure the concentration of DNA. DNA was sequenced at National Center for Genomic Research (Santa Fe, NM) using Illumina Hiseq version 2 chemistry.

Sequence assembly

MID barcodes were removed from each sequence and replaced with individual ID's using a custom Perl script. De novo assembly of sequences was conducted with a subset (11.2 million) of the total number of sequences (36 million) using SeqMan NGen 3.0.4 (DNASTAR). Of the 11.2 million reads used, 6.1 assembled. Next, contigs are pruned to remove sequences with too many insertion/deletions (indels), again a custom Perl script is used removing contigs with less than 82 bases or more than 90. A 'reference genome' is created from the denovo assembly so that all 36 million sequences can be assembled using a reference-based method. Reference based assembly was conducted using SeqMan xng 1.0.3.3 (DNASTAR) with merSize of 51bp and minimum match of 92%. This final assembly was then used to score variable sites.

Population genetic analysis

Variable sites are called using a custom Perl scripts, samtools and bfctools (Li *et al.* 2009). A minimum of 25% coverage at the site is required for it to be called as variable. We used an infinite sites model - so only two states are expected at each single nucleotide

polymorphism (SNP). After variant calling SNPs with more than 2 alleles were removed as well as those that did not conform to the expectations of the binomial distribution. This is because we are unable to distinguish between sites that have an extreme distribution and those that could have sequencing errors or bias in amplification. Next allele frequencies and genotype probabilities are estimated using a hierarchical Bayesian model (Gompert *et al.* 2012). The model incorporates both uncertainty in population level sampling and uncertainty in genotype (genetic sampling). We treat genotype at each locus and population level allele frequency as unknown parameters estimated from DNA sequence data. Probability distributions for both genotypes and allele frequencies were estimated separately for each population sampled. Posterior parameters for the model were estimated using Monte Carlo Markov Chain (MCMC) simulations, with 100,000 steps, a burn in of 10,000 and recording every 45th value. Only high-coverage loci, those with a minimum of 15 reads, were included for *N. menapia* populations. Two principal component analyses (PCA) were conducted using genotype probabilities, one for all populations and one for just *N. menapia* populations. Wrights F statistic (F_{ST}) (($H_T - H_S$) / H_T) was calculated from allele frequencies, as was heterozygosity. To visualize the relationships between pairwise F_{ST} values a non-metric multidimensional scaling analysis (NMDS) was conducted. All statistical analyses; PCA's, Wrights F_{ST}, heterozygosity, diagnostics for MCMC and NMDS were implemented in the statistical programming language R (http://www.**r**-project.org).

RESULTS

Wing Melanization

A graph was drawn showing the mean values of melanization (using residuals from regression of melanization on total wing area), including error bars around the mean (figure 3). Goat Mountain early brood (GE) and Mendocino Pass early brood (ME) have very

similar mean levels of melanization. The next closest population is Woodfords (WO) and then Oregon (OR). Furthest from GE and ME, are Donner Pass (DP) and Goat Mountain late brood (GL); these two populations have very similar mean melanization. With approximately median levels of melanization are Lang (LA) and Mendocino Pass late brood (ML). A one-way ANOVA conducted on melanization ~ population (table 3). Significant differences in melanization between different populations was found ($F_{7,188}$ = 41.12, P< 2e-167). Tukey's HSD was then carried out (figure 3), significant differences were found between sympatric early and late flights at both Goat Mountain and Mendocino Pass. Several other pairwise comparisons showed significant differences in melanization. Non-significant differences were found in 11 pairwise comparisons (out of 28).

Wing Shape

A one-way ANOVA on width:length ratio ~ population found significant differences in width:length ratio between populations ($F_{7.186}$ = 3.748, P = 0.00081)(table 4). Tukey's HSD post hoc test was used and found significant differences between early and late flights at just one of the sympatric sites, Mendocino Pass. There were also significant differences between Mendocino early flight population (ME) and Donner Pass (DP). All other pairwise comparisons were not significant (figure 4).

Population Genetics

The first principal component analysis (PCA) was conducted on all populations (figure 5); PC1 (26.04% variance explained) appears to divide populations based on their nominal species designation. *N. terloottii* was clearly distinguished from all populations of *N. menapia*. PC2 (7.9% variance explained) shows subdivision among the *N. menapia* populations with Coastal Mountain populations (Goat Mountain early and late, Mendocino Pass early and late and Oregon) clustering together away from Sierra Nevada sites (Donner Pass, Lang

and Woodfords). Another PCA was conducted using just *N. menapia* populations (figure 6). PC1 (10.79% variance explained) shows differences between Coastal Mountain and Sierra Nevada populations. PC2 (5.53% variance explained) shows further subdivision within the coastal mountain populations. Sympatric early and late flights at Goat Mountain cluster away from each other, at opposite ends of PC2 axis. Mendocino Pass early and late flights do not show the same level of genetic differentiation and stay closer together towards the center of PC2. The Oregon population clusters close to Mendocino Pass early and late brood populations.

Wrights F_{ST} was calculated from allele frequencies for all pairwise population comparisons (table 5). All pairwise comparisons had F_{ST} values significantly different from zero. F_{ST} between early and late flights at Goat Mountain is similar to F_{ST} between Goat Mountain populations and other, geographically isolated populations. At Mendocino Pass, F_{ST} between early and late flights is significantly different from 0 but relatively low compared to other F_{ST}'s. Overall, F_{ST}'s for all pairwise comparisons suggest recent isolation and/or ongoing gene flow. A non-parametric multi-dimensional scaling analysis was used to visualize the relationships between *N. menapia* populations using pairwise F_{ST} values (figure 7). Patterns of relatedness are similar to those seen in the PCA plots based on the individual genotype probabilities. The three Sierra Nevada sites cluster together (DP, LA and WO). Mendocino Pass early and late populations cluster towards the center, close to Goat Mountain early population. The late population at Goat Mountain clusters further away, again reflecting genetic differentiation between early and late flights at this site. Oregon clusters further away, but remains closer to the Californian Coastal Mountain populations relative to the Sierra Nevada sites.

DISCUSSION

Significant genetic and morphological differences were detected between early and late flights at both sympatric sites. The results were consistent with the hypothesis that early and late flights are allochronically isolated. Genetic differentiation was found between early and late flights at both of the sympatric sites, interestingly the patterns of differentiation are not the same at both of the sympatric sites. At Goat Mountain populations show higher levels of genetic differentiation relative to Mendocino Pass, as can be seen in the PCA (figure 6) of individual genotypes and the NMDS of pairwise F_{ST}'s (figure 7). On the graph of mean melanization (figure 3) differences between wings of early and late flights at Goat Mountain seem to be larger than differences between Mendocino Pass populations. Contrastingly, wing shape was significantly different between flights at Mendocino Pass but not between those at Goat Mountain (figure 4).

The variation observed between patterns of differentiation at the two sympatric sites could indicate that the process of allochronic isolation is variable. For example, the origin of allochronic isolation could be different; i.e. at one site an allochronically isolated population has arisen from within that site while at the other site colonization from an allopatric population with a later brood time may have occurred. Alternatively it may be that the two sites are different because isolation has arisen in sympatry at different times; Goat mountain populations may have been isolated from one another for longer than those at Mendocino Pass.

All pairwise F_{ST}'s calculated were significantly different from zero. All pairwise F_{ST}'s between *N. terloottii* and *N. menapia* populations were similar. Within *N. menapia* populations both the NMDS plot (figure 7) and PCA (figure 6) there is clear differentiation between

those populations from the Sierra Nevada and Coastal mountain ranges in both California and Oregon. This includes Oregon populations clustering with Coastal mountain sites in California despite considerable geographic isolation. Indicating that gene flow within ranges is more likely than between the Coast Range and the Sierra Nevada. Overall F_{ST}'s between *N. menapia* populations indicate that there has been recent isolation between populations and/or there is ongoing gene flow.

As previously mentioned, significant differences in wing melanization were found between early and late flights at both of the sympatric sites. The mechanism underlying melanization in this species remains unknown. In this study, the patterns of genetic differentiation between populations are not reflected in patterns of melanization. This suggests that melanization may not have underlying genetic basis or that our genomic sampling missed the regions of the genome associated with melanization. An alternative mechanism could be seasonal polyphenism (Shapiro 1976). If this is the case, differences in temperature during development could result in differences in melanization. Our results do not support this; melanization does not appear to be related to brood time or temperature across all populations (although records of average temperatures are not reliably available for all populations). Further work would be required to reliably distinguish the underlying mechanisms of melanization. Wing shape is known to vary among seasonal forms of pierid butterflies, in response to varying levels of wind (Shapiro 2007). At sympatric sites there appear to be differences in wing shape, this study only found significant differences at one of the sympatric sites, Mendocino Pass (figure 4). Wing shape was quantified in this study using a ratio of width to length; a more complete analysis may be required in order to capture the variation observed between early and late flights. Significant differences in wing shape may represent adaptation to flight time.

Patterns of genetic differentiation indicate that sympatric populations have arisen from the Coastal Range (second hypothesis), allowing us to exclude the Sierra Nevada sites as a source for the sympatric populations (first hypothesis). Further geographic sampling would be required to identify areas in the coastal region with the highest levels of genetic similarity to sympatric populations.

In conclusion, this study has discovered two cases of allochronic isolation in the pine-white butterfly, suggesting that allochrony is an important isolating mechanism for this species. Both genetic differentiation and morphological differences were found between early and late flights at the two sympatric sites. We determine the biogeographic origin of populations at the sympatric sites is likely to have come from within the coastal region, not from the Sierra Nevada. This case, along with other recent work on allochronic isolation (Abbot & Withgott 2004, Friesen *et al.* 2007, Yamamoto & Sota 2009, Ording *et al.* 2010, Santos *et al.* 2011a, Santos *et al.* 2011b, Yamamoto & Sota 2012), demonstrates that allochrony may occur more frequently than previously thought and warrants further research into the underlying mechanism of this process of reproductive isolation.

Table 1: Sampling Locations for *Neophasia menapia* and *Neophasia terloottii*

Site Location	Site Details	Elevation (ft.)	Number Collected	Brood Number	Collection Dates
Donner Pass, CA	Sierra Nevada	7,000	23	1	15.IX.99
Lang Crossing, CA	Sierra Nevada	4,528	20	1	25.VIII.95
Woodfords, CA	Sierra Nevada	5,617	22	1	25.VIII.95
Mendocino Pass, CA	Coast Range	5,000	26 in summer 20 in fall	2	18.VII.95, 30.VII.00, 18.IX.99, 22.IX.99
Goat Mountain, CA	Coast Range	3,655	24 in summer 26 in fall	2	22.VII.95, 18.IX.95, 01.X.95, 25.IX.99
Otis, OR	Coast Range	46	12	1	18.IX.99, 19.IX.99, 20.IX.99
Cochise County, Chiricahua and Huachuca mountains, AZ	*Neophasia terloottii* sampled from Arizonia will be used as an outgroup	-	14	-	02.XI.02, 26.VI.04, 06.XI.04

Table 2: Number of Male Wings Measured Per Population

Location	Melanization: Number of Males Measured	Wing Shape Ratio: Number of Males Measured
Donner Pass (DP)	25	25
Goat Mountain Early (GE)	30	29
Goat Mountain Late (GL)	31	30
Lang (LA)	14	14
Mendocino Pass Early (ME)	37	37
Mendocino Pass Late (ML)	18	18
Oregon (OR)	11	11
Woodfords (WO)	30	30

Table 3: One-way ANOVA of Melanization~Population

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Squares	F Ratio	P
Population	7	35171	5024	41.12	**<2 e-167**
Residuals	188	22972	122		

Table 4: One-way ANOVA of Wing Shape Ratio~Population

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Squares	F Ratio	P
Population	7	0.01991	0.002844	3.748	**0.000801**
Residuals	186	0.14117	0.000759		

Table 5: Pairwise Wright's F Statistic for all Populations: Top triangle 95% confidence intervals for pairwise Fst's, lower triangle pairwise Fst's

	AZ	DP	GE	GL	LA	ME	ML	OR	WO
AZ		0.449-0.456	0.446-0.453	0.451-0.458	0.446-0.452	0.439-0.446	0.440-0.447	0.447-0.455	0.442-0.449
DP	0.452		0.064-0.065	0.071-0.073	0.032-0.034	0.055-0.056	0.058-0.060	0.075-0.077	0.039-0.040
GE	0.449	0.054		0.056-0.057	0.059-0.060	0.038-0.039	0.043-0.044	0.061-0.063	0.054-0.055
GL	0.454	0.07	0.057		0.066-0.068	0.043-0.044	0.041-0.043	0.066-0.068	0.062-0.063
LA	0.448	0.033	0.060	0.067		0.050-0.051	0.053-0.054	0.071-0.072	0.035-0.036
ME	0.442	0.055	0.038	0.043	0.051		0.030-0.031	0.052-0.054	0.044-0.046
ML	0.443	0.059	0.044	0.042	0.054	0.030		0.054-0.055	0.048-0.049
OR	0.451	0.076	0.062	0.067	0.071	0.053	0.055		0.066-0.068
WO	0.449	0.063	0.054	0.063	0.035	0.045	0.049	0.067	

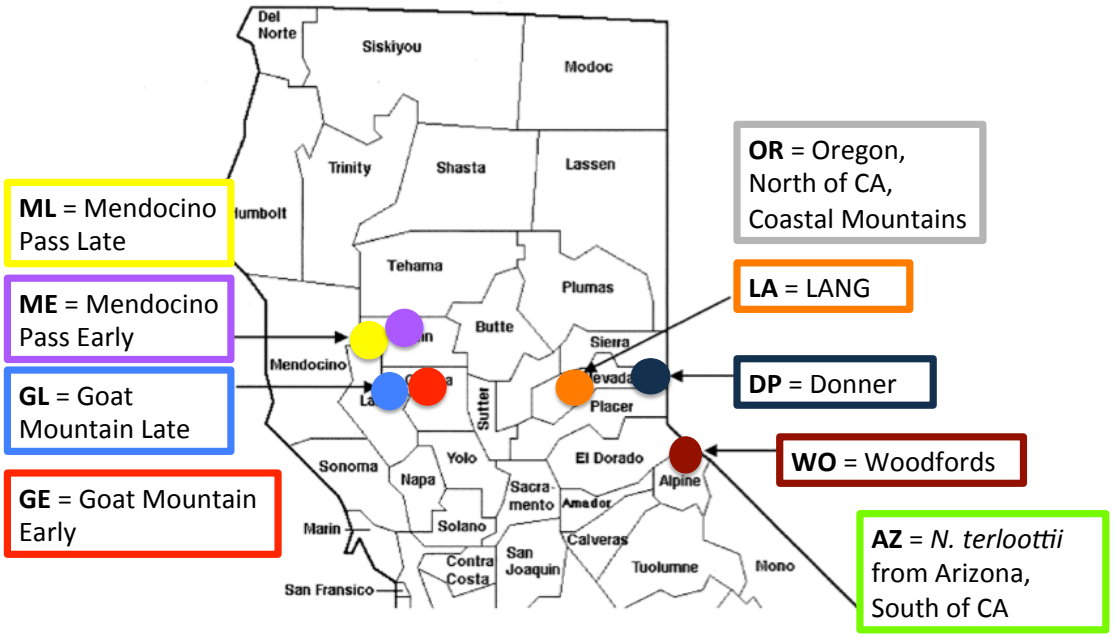
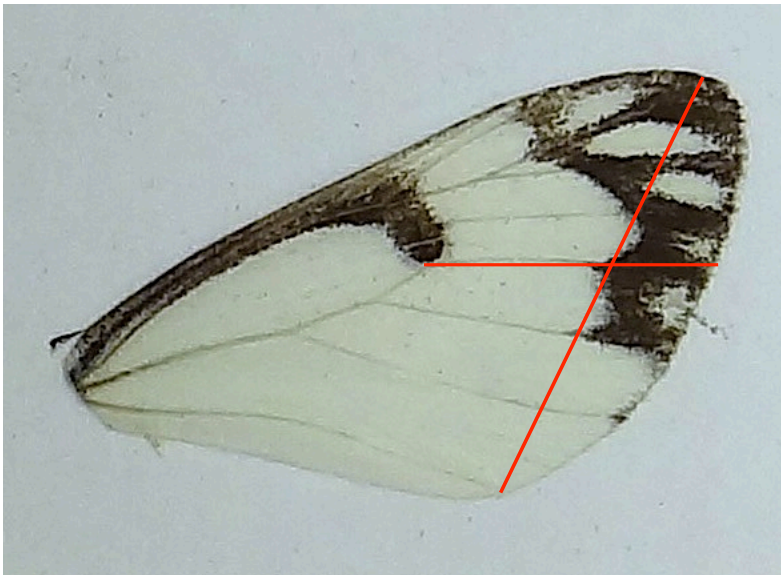


Figure 1: Map of Sampling Locations in California



Measurement 1: taken from where subcoastal vein meets the wing margin to where the anal vein meets the wing margin

Measurement 2: taken from vein M3 from discal cell to where it meets the wing margin

Figure 2: Wing Shape Measurement

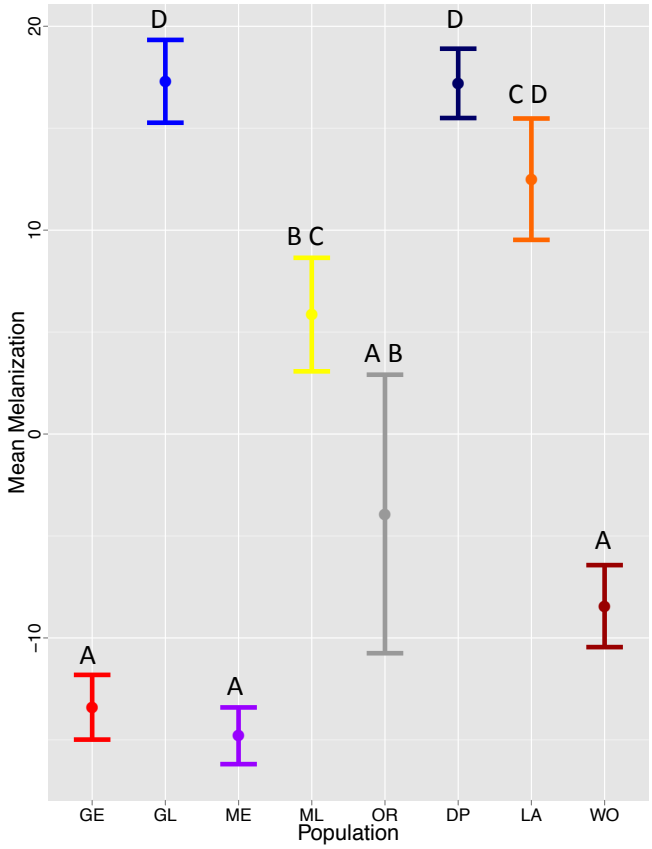


Figure 3: Graph Showing Mean Melanization Per Population

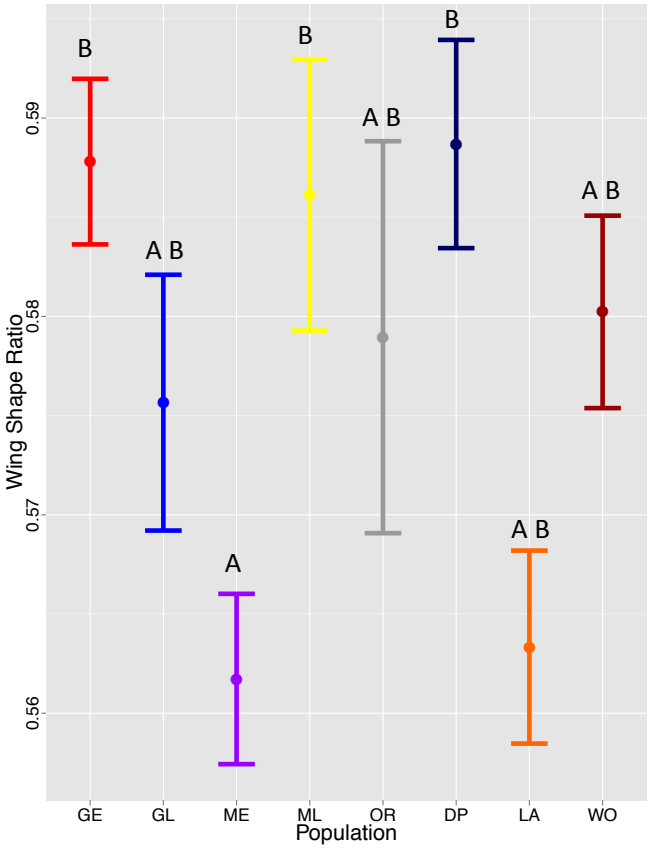


Figure 4: Graph Showing Mean Wing Shape Ratio Per Population

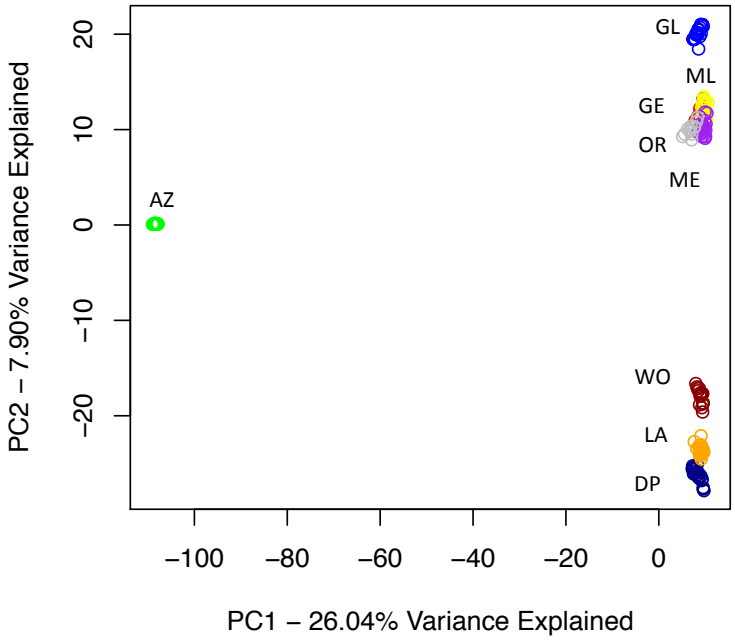


Figure 5: Principal Component Analysis (PCA) of Genotype Probabilities for All Populations

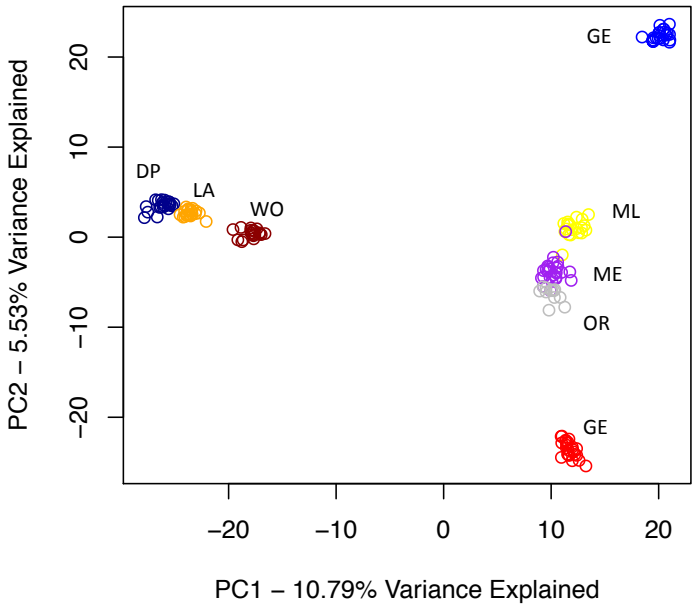


Figure 6: Principal Component Analysis (PCA) of Genotype Probabilities for *Neophasia menapia* Populations

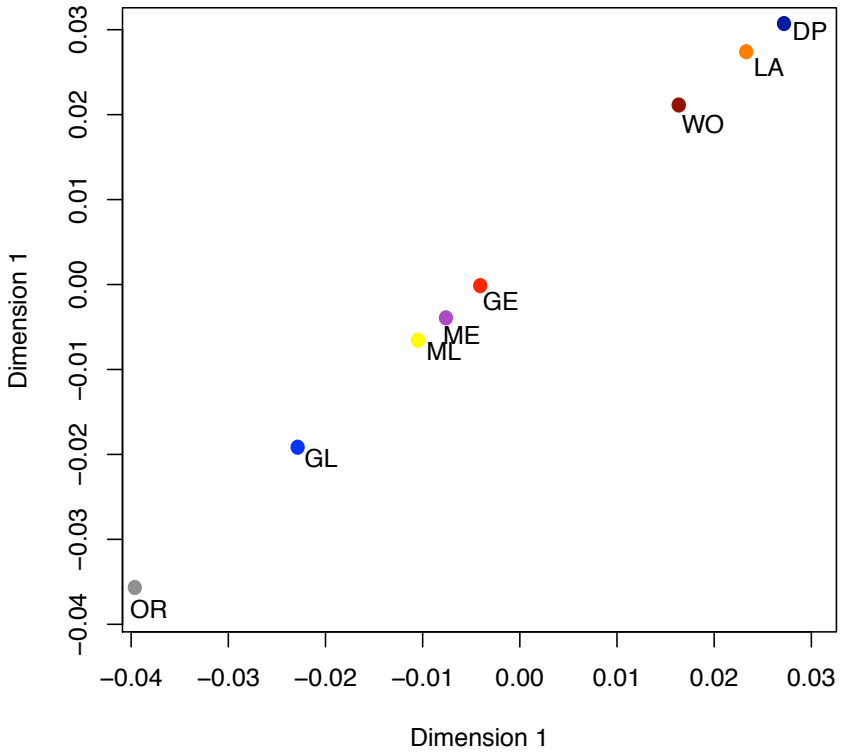


Figure 7: Non-metric Multi Dimensional Scaling (NMDS) plot of Pairwise F_{ST}'s for *Neophasia menapia* Populations

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