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Sympatric, Temporally Isolated Populations of the Pine White Butterfly *Neophasia menapia*, are Morphologically and Genetically Differentiated

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Abstract

Temporal isolation remains an understudied, and potentially under-appreciated, mechanism of reproductive isolation. Phenological differences have been discovered in populations of the pine white butterfly (*Neophasia menapia*), a typically univoltine species found throughout western North America. However at two locations in the Coast Range of California there are two periods of adult emergence per year, one in early summer (July) and one in late summer/autumn (September/October). Differences in flight time are accompanied by differences in wing shape and pigmentation. Here we use a combination of population genomics and morphological analyses to assess the extent to which temporal isolation is able to limit gene flow between sympatric early and late flights and to explore several potential hypotheses about the origin of these sympatric flights. We detected significant genetic differentiation between early and late flights and test whether these populations originated *in situ* or resulted from one or more colonization events.

Introduction

The study of the origin and maintenance of reproductive isolation remains a central focus in evolutionary biology and provides key insights into the process of speciation. Variation in phenology, the seasonal timing of life history events, can act as a reproductively isolating mechanism. Our knowledge of the evolutionary consequences of this isolation, specifically its role in diversification, is relatively incomplete (Abbot & Withgott, 2004). Phenological differences may arise in response to other diversifying mechanisms. For example, environmental change, geographic isolation, or a shift in resource use may drive the evolution of phenology (Feder et al., 1993, 1994). In many cases temporal isolation is considered to reinforce reproductive isolation, rather than to be the primary isolating mechanism. The term allochronic speciation was developed to describe cases in which the initial stages of speciation are set in motion by a change in phenology (Alexander & Bigelow, 1960; Abbot & Withgott, 2004). Once thought to be a relatively rare form of reproductive isolation, in recent years there have been examples of allochronic and temporal isolation across many diverse taxa; including insects (Santos et al., 2007; Yamamoto & Sota, 2009, 2012; Ording et al., 2010), plants (Devaux & Lande, 2009), birds (Friesen et al., 2007) and corals (Tomaiuolo et al., 2007), indicating that temporal differentiation is a potentially important isolating mechanism.

While temporal differentiation can facilitate divergence and speciation, regulation of activity and phenology typically results in synchronization of behavior within populations or species. Many factors may contribute to synchronization. For phenological synchronization in insects, one such strategy is diapause, a quiescent state in which annual periods of unfavorable climate are bypassed (Scott, 1992). Shifts in phenology have been well documented, especially in insects, and often involve changes in diapause (Thomas et al., 2003). Diapause is wide spread among the Class Insecta. It can occur at diverse embryonic stages, from eggs through to adults, but within a species it's typically restricted

to a single stage (Denlinger, 2002). Faculative is the most frequent form of diapause, this occurs when the timing of diapause is mediated by environmental cues - the most common of which is day length (Denlinger, 2002). When phenological shifts occur, presumably due to disruptive or divergent selection, synchronization within populations can reinforce divergence between populations. This temporal divergence can occur in sympatry or in allopatry that might be followed by range changes that bring the diverging populations into sympatry. We are interested in whether, and to what extent, these temporal, life history changes restrict geneflow.

Here we investigate a possible case of temporal isolation in *Neophasia menapia*, the pine white butterfly, which occurs throughout western North America (Scott, 1992; Guppy & Shepard, 2001). The common name refers to the use of various pine species (Pinaceae) as the larval host (Guppy & Shepard, 2001). The pine white is a univoltine species; adults emerge in summer, eggs are laid and overwinter (enter diapause) until the following spring when they hatch. Caterpillars feed on pine needles and develop directly, pupate, and adults emerge, mate and lay eggs that diapause the following winter (Fletcher, 1905; Elrod & Maley, 1906; Comstock, 1924; Garth, 1930; Belvins & Belvins, 1944; Brock, 2006). In California, two locations in the Coast Range 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 two sites, differences in emergence time, early or late, appear to be accompanied by differences in wing morphology with the late flight appearing to have more melanization and broader wings than the early flight. The sympatric nature of these populations provides a novel opportunity to study changes in phenology without the confounding factor of contemporary environmental variation.

We use a combination of population genomics and morphological analyses to examine the extent to which these sympatric early and late flights in the Coast Range are differentiated and isolated and to test hypotheses on the possible origin of these sympatric

flights. We address three specific questions: 1). Do sympatric early and late flights exhibit population genomic differentiation consistent with the hypothesis of temporal isolation? If no genetic differentiation is detected, this would be consistent with the alternative hypothesis that *N. menapia* populations at these sites have undergone a shift in life history to become bivoltine (two generations per year). If this is the case there would be no reproductive isolation as the early flight population would be the parental population to the late flight. 2). How different are wing pigmentation and wing shape between the two sympatric flights at each of the sites, and compared to other nearby *N. menapia* populations? 3). What can we infer about the origin(s) of the sympatric populations? There are several hypotheses on the origins of sympatric populations: firstly, a single invasion of the Coast Range occurring from one (or more) of the nearby sites. Secondly, sympatric early and late flights could have arisen via colonization from within the Coast Range, or these flights could have arisen *in situ*. A combination of high resolution, multi-locus genomic data and morphometric analyses was used to address these questions.

Methods and Materials

Butterfly Biology

The genus *Neophasia* (Pierinae) includes only two species worldwide, both occurring in North America. The common name of Pine White butterflies refers to their use of host plants from the Pinaceae family (pines, furs and hemlocks) (Guppy & Shepard, 2001), *Neophasia menapia* occurs throughout western North America while the second species, *Neophasia terlootii* occurs in southwestern USA and northwestern Mexico (Guppy & Shepard, 2001).

The wings of *N. menapia* are white with strong black markings around the leading edge of the forewing that curves around to form a cell-end bar (Brock, 2006; Glassberg,

1999). There are black markings along the veins of the hind wings in both males and females (Evenden, 1926). Some females may have bright orange-red markings along the apical margin of the underside hind wing (Evenden, 1926).

Throughout their range *N.menapia* are univoltine, meaning they have one flight per year (Guppy & Shepard, 2001; Scott et al., 1986; Layberry et al., 1998; Ferris et al., 1981; Shapiro et al., 2007; Marrone, 2002; Garth & Tilden, 1986). They are known to fly from late July until early September, and are most common in August (Fletcher, 1905; Elrod & Maley, 1906; Garth, 1930; Comstock, 1924). It has been suggested that elevation may affect the time of flight, with earlier flights (July) occurring at low elevations and later flights (September) occurring at high elevations (Shapiro et al., 2007; Guppy & Shepard, 2001). Females lay eggs in rows along pine needles in groups of up to 40, they overwinter (diapause) as eggs, and larvae begin feeding in Spring (Shapiro et al., 2007; Guppy & Shepard, 2001).

To the best of our knowledge, *N. menapia* is not known to exhibit wing pattern polyphenism (seasonal or otherwise), nor is there any evidence of multiple generations. Unfortunately, females fail to oviposit in laboratory settings (A.M. Shapiro, pers. obs.) which prevents manipulative experimental approaches to investigating the mechanisms of phenotypic differentiation. Therefore, we have approached the study of differentiation from a geographical, comparative perspective.

Sampling and Collection

A total of 187 butterflies were collected between 1995 and 2002 at several locations across California, Arizona and Oregon (Table 1). We collected 173 *N. menapia* at five sites in California, and one site in Oregon (Figure 1). At both Goat Mountain and Mendocino Pass in the Coast Range, two flights, early and late, have been observed. At these sites individuals were collected during both periods of adult flight, resulting in an early and a

late group for both sampling locations. The extent to which these two flights are locally sympatric is not clear, thus it is uncertain what role environmental factors play in determining phenological differences. The late flights at both Goat Mountain and Mendocino Pass seem to be more associated with west-facing slopes, whereas the early flights are more commonly collected on east-facing aspects. Individuals at each flight have been collected in close proximity, albeit at very different times, and the butterflies are certainly capable of flying across the entire area where the two flights are encountered. We consider the early and late flights at Goat Mountain and Mendocino Pass to be broadly sympatric. Beyond the Coast Range, three sites in the Sierra Nevada were sampled: Lang Crossing, Woodfords and Donner Pass (Figure 1). All locations sampled in Sierra Nevada were univoltine (one generation/flight per year). In Arizona 14 *N. terloottii*, the only other species in the genus, were sampled and included as a basis for comparison in the analysis of population structure of *N. menapia*. All samples were kept at -80°C until DNA extraction.

Molecular Methods

Next generation DNA sequence data were generated following Gompert et al. (2012) and Parchman et al. (2012). DNA was isolated and purified from each sampled butterfly from approximately 0.1 grams 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). We fragmented DNA using two restriction enzymes (EcoR1 and Mse1) resulting in a genomic DNA library for each individual. Customized Illumina adaptor sequences and an eight to ten base pair MID (multiplex identifier) barcode were ligated to DNA fragments for each individual. Two rounds of PCR were used to amplify individual libraries, after which PCR products were pooled across all individuals. This resulted in a pooled library for 187 individuals, with fragments identifiable by unique 10bp barcodes. Pooled PCR products were separated on a two percent agarose gel and fragments between 300-500bp were selected by excising them from

the gel using QIAquick gel extraction kit (QIAgen Inc.) as per the manufacturer's protocol. DNA was sequenced at the National Center for Genomic Research (Santa Fe, NM) using Illumina HiSeq version 2 chemistry.

We obtained 36 million sequence reads which were processed using a series of quality control steps to identify variable sites, following the methods of Gompert et al. (2012). In overview, custom perl scripts were used to identify sequences to an individual based on barcode sequences. We then removed barcodes and removed sequences that contained adaptor sequence or that were of poor quality. De novo assembly was conducted on a subset of reads (11.2 million) using Seqman Ngen 3.0.4 (DNASTAR). Consensus sequences from the assembly were concatenated to produce an artificial chromosome for reference-based assembly of the total 36 millions reads using Seqman Ngen 3.0.4 (DNASTAR). Variable sites were called using custom Perl scripts, SAMtools and bcftools (Li et al., 2009). A minimum of 25 percent coverage at a site was required for the site to be called as variable. We assumed an infinite sites model, thus all variable sites with more than two nucleotides (alleles) were removed. This resulted in 40,389 variable sites.

Population Genetic Analyses

Data were trimmed to only include Single Nucleotide Polymorphisms (SNPs) with a minimum of 15 reads per population sample, producing 20,737 SNPs. We used the allele frequency model presented in Gompert & Buerkle (2011) to estimate allele frequencies for each locus based on the observed data; this is a similar approach to that used by Pritchard et al. (2000), Gillespie (2004) and Hedrick (2005). The model treats genotypes and allele frequencies as parameters that are estimated from the sequence data. For a more detailed description see Gompert & Buerkle (2011) and Parchman et al. (2012). The posterior probabilities of parameter estimates (allele frequencies per population and genotype probabilities per locus per individual) were obtained using Markov Chain Monte Carlo (MCMC) with 100,000 steps and a burn-in of 10,000.

Genetic structure at the individual level was summarized using a principal component analysis (PCA) and the admixture model in STRUCTURE 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). The PCA was conducted using genotype posterior probabilities for the 3 genotypes at each SNP (20,737) for each individual, using the statistical program R (using the prcomp function in the composition package in R). We produced two PCA's, one that includes both nominal species, *N. terloottii* and *N. menapia*, and a second PCA using only *N. menapia* populations. For the analysis using the program STRUCTURE, we sampled one sequence read for each SNP locus for each individual in proportion to the frequency of reads at that locus for each individual. Thus individuals were assigned either a 1 or a 2 depending on which sequence read was sampled for that individual and -9 (missing data) for the alternative allele for each locus (script written by T. Parchman, University of Nevada, Reno). Our infile is similar to that used for dominant markers where heterozygosity at a locus cannot be verified. Individuals with more than 98 percent missing data were removed (1 individual from *N. terloottii* population, 4 individuals from Goat Mountain late population sample). For the STRUCTURE analysis 19,152 SNPs were included. The admixture model was used to estimate admixture proportions of each of K groups. Again, two analyses were conducted, one that included both nominal species and one that included just *N. menapia* populations. The model was run for K=1-12 (number of putative populations + 3) and K=1-11 respectively, with 10 runs per K. Monte Carlo Markov Chain (MCMC) procedures were used to obtain estimates, with 100,000 steps and a burn in of 50,000 steps. To estimate the appropriate K (number of groups) the log of the marginal likelihood (Pritchard et al., 2000) was plotted against K and the ad hoc Δ K statistic was calculated and plotted against K (Evanno et al., 2005). At the population level we calculated pairwise G_{ST} statistics among all populations from allele frequency estimators (Nei, 1973). G_{ST} estimates were summarized using a non-metric multidimensional scaling (NMDS) conducted in R using the package MASS.

Geometric Morphometrics

To assay variation in wing pigment patterns (melanization) and wing shape forewings of male *N. menapia* were photographed using a digital camera (Sony Cyber-shot HX9V) on a white background with a scale (mm ruler) (Table 2). As our sample included more males than females, we used only male wings in order to avoid complications from sexual dimorphism. Measurements were taken for the left forewing unless there was wing damage, in which case, the right wing was used. Specific damage to a wing could lead to the exclusion of that sample from either the wing pigment analysis or the wing shape analysis, leading to differing samples sizes between the two approaches.

Wing Melanization All measurements for wing melanization were taken using IMAGEJ software (Schneider et al., 2012). The area of each wing was measured twice and the average of the two measurements was used in all analyses. Images were transformed to grey scale and then made binary, allowing the total area of black on the wing to be measured. 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 using the function glm in R (R Core Team, 2015), and the residuals used in further statistical analysis in order to remove the influence of wing area on total melanization. A one-way ANOVA followed by Tukey's HSD was used to examine which populations differed significantly in wing melanization (R Core Team, 2015).

Wing Shape

We identified 12 landmarks, located either at convergence points between wing veins or the intersection of a vein and the edge of the wing (Figure 2). X,Y, co-ordinates of the landmarks were measured using IMAGEJ software. Co-ordinates were imported into MorphoJ for further analyses (Klingenberg, 2011). A generalized procrustes analysis, which removes non-shape variation such as rotation and scale, was used to normalize co-ordinates

(Rohlf, 1999). In order to control for allometry (variation in shape because of size), a multivariate regression of wing shape (dependent variable) on centroid size (independent variable) was conducted in MorphoJ software (Klingenberg, 2011). Centroid size is an isometric estimator of size calculated by taking the square root of each summed square distance of each landmark from the center of the landmark configuration (Bookstein, 1991). The residuals of this regression were used in all subsequent analyses. To identify the main axes of variation within the data set we conducted a principal component analysis, using a covariance matrix in MorphoJ. We then carried out three ANOVA's, one using PC1 scores, a second using PC2 scores and finally one with PC3 scores. A Tukey's HSD post hoc test was then used to examine which pairwise comparisons were significantly different. We also used a canonical variate analysis (CVA) to explore patterns of variation among groups. In this analysis groups are identified *a priori* and canonical variables are calculated that maximize the amount of among group variance relative to within groups. This allows for visualization of the variation among groups. For both the PCA and the CVA, 95% confidence ellipses around the mean, using population as a classifier, were plotted. For CV1 and CV2 a transformation grid plot showing wing shape changes was plotted in MorphoJ (Klingenberg, 2011).

Results

Population Genetics

We used approximately 20,000 SNPs (20,737 SNPs for PCA and G_{ST}, 19,152 SNPs for STRUCTURE analysis) obtained from assembly of 36 million Illumina sequence reads. A principal component analysis (PCA) was conducted on all eight *N. menapia* sample groups and the one group of *N. terloottii* (Figure 3A). PC1 explained 26.04% of the variance and divided groups based on their nominal species designation. *N. terloottii* is clearly

distinguished from all *N. menapia* populations. PC2, which explained 7.9% of the variance, showed subdivision among the *N. menapia* population samples, with Coast Range populations (Goat Mountain early and late, Mendocino Pass early and late and Oregon) clustering together, separate from Sierra Nevada sites (Donner Pass, Lang and Woodfords). A second PCA was conducted to explore patterns of differentiation among the *N. menapia* samples (Figure 3B). PC1, which explained 10.79% of the variance, separated Coast Range and Sierra Nevada samples while PC2, which explained 5.53% of the variance, showed further subdivision within the Coast Range populations. Sympatric early and late flights at Goat Mountain clustered separately, at opposite ends of PC2 axis. Mendocino Pass early and late flights did not show the same level of genetic differentiation and were closer together towards the center of PC2. The Oregon population clustered close to Mendocino Pass early and late flight populations.

All pairwise comparisons resulted in G_{ST} values significantly different from zero (Table 3). Pairwise G_{ST} comparisons between each *N. meanpia* sampling location and *N. terloottii* were of a similar scale and higher than any of the intraspecific comparisons. G_{ST} between early and late flights at Goat Mountain was similar to G_{ST} between Goat Mountain and other, geographically isolated populations. At Mendocino Pass, G_{ST} between early and late flights was significantly different from zero but was relatively low compared to other G_{ST}'s. A non-parametric multi-dimensional scaling analysis (NMDS) was used to visualize the relationships between *N. menapia* sampling groups using pairwise G_{ST} values and showed patterns of relatedness (Figure 4) similar to those seen in the PCA plots based on the individual genotype probabilities. The three Sierra Nevada sites clustered together (Donner Pass, Lang and Woodfords). Mendocino Pass early and late populations clustered relatively close together while the early and late flights at Goat Mountain clustered at opposite ends of dimension three reflecting genetic differentiation between early and late flights at this site. The Oregon sample is distinct, but remains closer to the Californian Coast Range populations relative to the Sierra Nevada sites.

In the first STRUCTURE analysis that included both species, K =2 or K=3 were found to be the best clustering solutions. When assignment probabilities were plotted for K=2, *N. terloottii* formed one cluster, while the *N. menapia* samples formed a second cluster (Figure 5A). For K=3, *N. terloottii* formed the first cluster, then *N. menapia* populations split into two clusters, populations from the Coast Range and populations from Sierra Nevada (Figure 5B). For *N. menapia*, K was found to be either 4 or 5. When assignment probabilities for K=4 were plotted the three Sierra Nevada sites group together, early and late flights at Goat Mountain formed two separate clusters, early and late flights at Mendocino Pass formed an apparently admixed group and the Oregon sample formed its own cluster but with some assignment to the Mendocino Pass cluster (Figure 6A). For K=5, the groups stay the same but Oregon forms its own cluster, distinct from the two Mendocino groups (Figure 6B).

Geometric Morphometrics

Wing Shape

Mean values of melanization (plus or minus standard error) were plotted for each sampling group (Figure 7). Goat Mountain early flight and Mendocino Pass early flight have very similar mean levels of melanization. The next closest group is Woodfords and then Oregon. Furthest from the two early flights are Donner Pass and Goat Mountain late flight; these groups have similar mean melanization. With approximately intermediate levels of melanization are Lang and Mendocino Pass late flight. A one-way ANOVA was conducted to explore variation in melanization between populations (Table 4). Significant differences in melanization per population were found ($F_{7,188} = 41.12$, P< 2e-167). A post hoc test, Tukey's HSD test, was carried out to identify which pairwise comparisons were significantly different. 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 PCA was carried out on the 12 landmarks to identify the main axes of variation in wing shape. When PC1 (24.45% variance explained) and PC2 (15.48% variance explained) are plotted there appears to be little discernible clustering by sampling group (Figure 8A). 95% confidence ellipses around the mean for each population sample show overlap between several populations but not between the early and late flights at either Goat Mountain or Mendocino Pass. To test statistically for differences between groups and their PC scores, a one way ANOVA was used with Tukey's HSD post hoc test to identify which pairwise comparisons were significantly different. For both PC1 and PC2 scores, significant differences were found between early and late flights at Goat Mountain, but not for PC3. At Mendocino Pass there were significant differences between early and late flights for their PC2 scores.

To further explore patterns of variation among groups, a CVA was used. CVA differs from a PCA because groups are assigned *a priori* and the analysis maximizes among-group differences relative to within-group differences. In a plot of CV1 (47.65% variance explained) and CV2 (16.07% variance explained), Goat Mountain early flight sample clusters towards the far end of CV1 away from the late flight at Goat Mountain, the same pattern can be seen for Mendocino Pass early and late flight groups (Figure 8B). The 95% confidence ellipses demonstrate differences in the mean between early and late flights at both sites. The three Sierra Nevada populations cluster relatively close together but do not overlap. Transformation grid plots show that for CV1 there are noticeable shifts in landmark 1 and landmark 7 as well as slight changes in several other landmarks. For CV2 there are also changes in landmarks 1 and 7 as well as changes in landmark 8 (Figure 9). As was found with wing melanization, differences in wing shape were found between

early and late flights, but this variation falls within the variation seen between other sampling locations.

Discussion

We used a genome-wide survey of DNA sequence variation and morphological analyses of wing shape and pigmentation to explore the evolutionary significance of sympatric early and late flights of *N. menapia* at two locations in California. Our data were used to test the hypothesis of temporal isolation between sympatric early and late flights and examine various hypotheses about their origin. We found significant genetic and morphological differences between sympatric early and late flights of *N. menapia* at both sites in the California Coast Range. Interestingly, patterns of genetic differentiation were variable among the two sites, with Goat Mountain early and late flights showing higher levels of differentiation than early and late fights at Mendocino Pass. Patterns in wing morphology were also variable between the two sites. However, patterns of genetic structure and morphological structure are not congruent. We found little evidence that the flights originated from an allopatric population in the Sierra Nevada and conclude that they either originated from within the Coast Range, or from an un-sampled allopatric population.

To return to our initial research questions; we first wanted to explore the population genomics of sympatric early and late flights and identify if there were levels of genetic structure present that would be consistent with the hypothesis of temporal isolation. Our results provide support for the hypothesis of temporal isolation between sympatric early and late flights at both locations. At Goat Mountain, populations show higher levels of genetic differentiation relative to Mendocino Pass, as can be seen in the PCA (Figure 3) of individual genotypes and the NMDS of pairwise G_{ST}'s (Figure 4). At Goat Mountain differentiation between early and late populations is at a similar scale to differentiation between geographically isolated populations located in different mountain ranges (Sierra

Nevada vs. Coast Range) (Table 3). At Mendocino Pass differentiation was not as great as that observed at Goat Mountain, but the G_{ST}'s calculated between early and late flights was significantly different from zero. This provides strong evidence against our alternative hypothesis that *N. menapia* populations have switched from a univoltine (one generation per year) to a bivoltine (two generations per year) life cycle. If populations had become bivoltine we would not expect to identify any significant genetic differentiation as early flight individuals would represent the parental populations of late flight individuals (or vice versa).

Our second question asked if sympatric early and late flights differed from each other, and other allopatrically isolated *N. menapia* populations in wing pigmentation (melanization) and wing shape. These morphological traits were chosen based on field observations and represent a preliminary assessment of potentially adaptive differences between early and late flights. Significant differences in both wing melanization and wing shape were found between sympatric early and late flights at both Goat Mountain and Mendocino Pass (Figure 7 & 8). An ANOVA found significant differences in melanization between early and late flights at both sites, as well as between pairwise comparisons of several other allopatric sites (Figure 7). For wing shape we found several significant differences between populations using an ANOVA. As with melanization, there were differences between early and late flights, and among several other comparisons. Patterns of wing shape differentiation did not reflect either the patterns seen in melanization or the genetic patterns identified. The CV_1 axis appears to divide early vs. late flights, while CV_2 divides populations based on sampling location (Figure 8). We found no overlap in the 95% confidence ellipses of the mean, for early and late flight populations (Figure 8). The mechanism underlying variation in melanization and wing shape in this species remains unknown. Increased melanization on the distal portion of the forewing is unlikely to play a thermoregulatory role, and to our knowledge there is no evidence of wing polyphenism in this species. However, it is certainly possible that the morphological differentiation among

the sampled populations is attributable to plasticity in response to environmental differences, at least in part. This research does not address the likelihood that morphological differences are the result of plasticity or genetic changes, but aims to take the initial step of quantifying differences. Irregardless of the underlying basis of wing morphology in this species there is the potential that the observed morphological patterns could represent adaptive evolutionary change (Fitzpatrick, 2012). Further research would be required to assess the underlying basis of these traits, and the possible evolutionary significance of this variation.

Our final question aimed to explore hypotheses about the possible origins of sympatric early and late flights. The genetic differentiation seen among *N. menapia* populations is not at the same scale as that between *N. menapia* and its sister species *N. terloottii*. This indicates that isolation between *N. menapia* populations is relatively recent and/or there is ongoing gene flow to some extent. Two alternate hypotheses about the origin of early and late flights involve either colonization occurring from one (or more) Sierra Nevada sites or that sympatric flights have arisen from within the Coast Range. We have found no support for the first hypothesis, colonization from an allopatric Sierra Nevada population. In terms of genetic differentiation the NMDS plot (Figure 4), PCA (Figure 3) and STRUCTURE assignment probability plots (Figure 6) demonstrate that there is clear differentiation between populations from the Sierra Nevada and Coast Range. This includes Oregon clustering with Coast Range 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. Further geographic sampling is required to identify areas in the Coast Range that could be the source of colonists to either the early or late flight. We know of no other localities with sympatric, phenologically isolated flights of *N. menapia*, however, Shapiro et al. (1979) noted phenological differences between populations of *N. menapia* in the Trinity Alps in northwestern California. There, butterflies at lower elevations (900m) fly earlier (June - July) and higher elevation (1500m)

butterflies appear later (September - October), but without the phenotypic differentiation observed at Goat Mountain and Mendocino Pass. Similar phenological isolation has also been noted for other species of butterflies. For example, Shapiro & Forister (2005) described phenologically isolated populations of skippers in the *Hesperia colorado* complex, with the later-flying population at one sympatric site being associated with serpentine soils. However, the causes of phenological isolation in that case, as with *N. menapia*, remain mysterious. Furthermore, we are not presently able to identify if sympatric early and late populations of *N. menapia* arose *in situ* or if there has been a colonization event from another Coast Range population that was not included in our sampling.

Although population genetic differentiation has been identified between sympatric populations at both sites, the extent of differentiation is not the same. Variation between the two sites could indicate that the process of temporal isolation is variable. For example, the origin of temporal isolation could be different; i.e. at one site a temporally isolated population has arisen *in situ*, while at the other site colonization from an allopatric population with a later flight 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. Morphological measurements, wing melanization and wing shape (Figures 7 & 8), do not reflect these genetic patterns and are not consistent with one another in terms of structure among populations. Given that the genetic basis of these traits is unknown for this species, it would be inappropriate to infer evolutionary relationships based on these data.

In order to explore these unanswered questions, and other evolutionary details of these temporally isolated sympatric populations, further research is required. For example, further geographical sampling, lab-based experiments to examine variation in the dynamics and control of diapause, especially the termination of diapause, or exploration of the potential adaptive significance of wing morphology would expand our understanding of the

evolutionary significance of this temporal isolation.

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

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Tables and Figures

Table 1: Sampling locations for *Neophasia menapia* and *Neophasia terloottii* used in genomic analyses. Number in parentheses after each collection date represents the number of individuals collected in that year.

Species	Site Location	Abbreviation	Site Details	Elevation (ft.)	Number Collected	Collection Date
N. menapia	Donner Pass, CA	DP	Sierra Nevada	7,000	23	September '95
	Lang Crossing, CA	LA	Sierra Nevada	4,528	20	August '95
	Woodfords, CA	WO	Sierra Nevada	5,617	21	August '95 (16), '00 (5)
	Goat Mountain early flight, CA	GE	Coast Range	3,655	24	July '95
	Goat Mountain late flight, CA	GL	Coast Range	3,655	26	October '95 (18), September '99 (8)
	Mendocino Pass early flight, CA	ME	Coast Range	5,000	26	July '95 (15), '00 (11)
	Mendocino Pass late flight, CA	ML	Coast Range	5,000	20	September '95 (18), '99 (2)
	Otis, OR	OR	Coast Range	46	12	September '00
N. terloottii	Cochise County, AZ	AZ	Chiricahua, Huachuca mountains	9,500	14	October '91 (4), November '02 (4), '04 (4)

Table 2: Number of male *N. menapia* wings measured per population sample in wing morphology analyses.

Location	Melanization	Wing Shape
Donner Pass (DP)	25	23
Lang (LA)	14	14
Woodfords (WO)	30	29
Goat Mountain Early (GE)	30	40
Goat Mountain Late (GL)	31	42
Mendocino Pass Early (ME)	37	40
Mendocino Pass Late (ML)	18	20
Oregon (OR)	11	14

Table 3: Pairwise G_{ST}'s calculated from allele frequencies: lower triangle G_{ST} estimate, top triangle 95% credible intervals.

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

Table 4: One-way ANOVA of melanization area by sampling location

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		

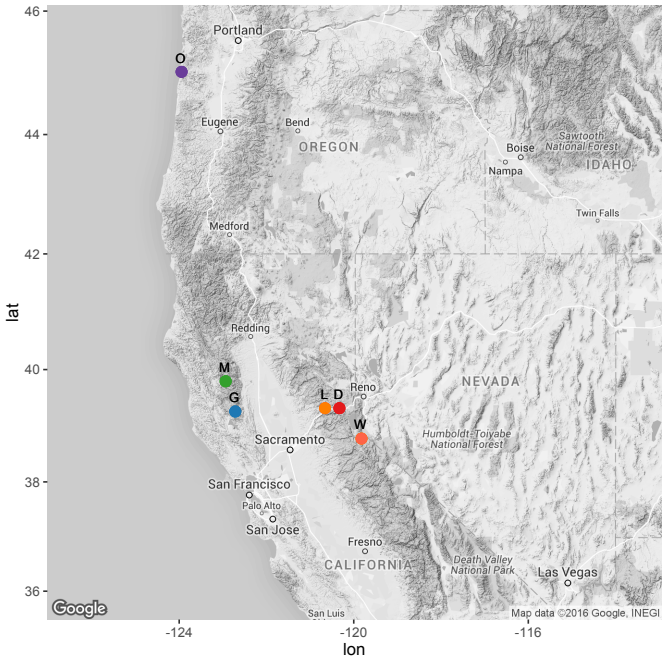

Figure 1: Map of *N. menapia* sampling locations; D (red) = Donner Pass, L (orange) = Lang, W (light red) = Woodfords, G (dark blue) = Goat Mounain, M (dark green) = Mendocino Pass late flight, O (purple) = Oregon. *N. terloottii* were sampled from Arizona, not shown on the map

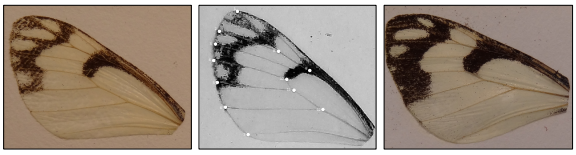

Figure 2: Left panel: male forewing from Goat Mountain early flight. Middle panel: location of 12 landmarks on *N. menapia* forewing, wing changed to greyscale in ImageJ. Right panel: Male forewing from Goat Mountain late flight.

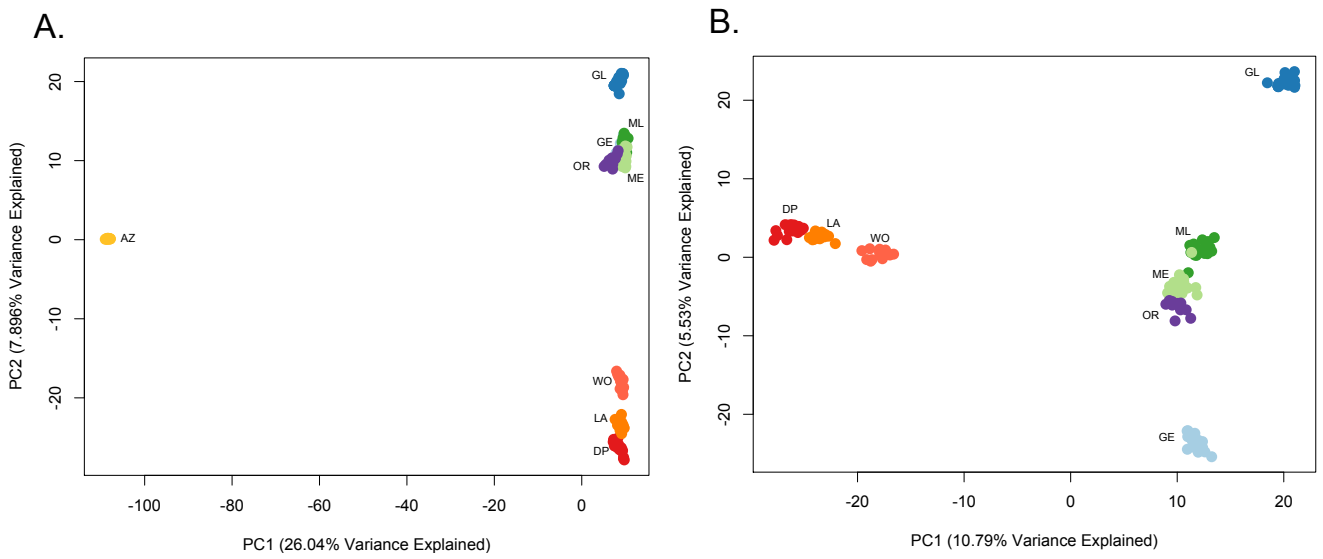

Figure 3: PCA based on genotype probabilities where each circle represents an individual's genotype probabilities across all 20,737 SNPs; A: PCA for *N. terloottii* and *N. menapia* B: PCA for *N. menapia* population samples; AZ (yellow) = *N. terloottii* from Arizonia, *N. menapia* samples from; DP (red) = Donner Pass, LA (orange) = Lang, WO (light red) = Woodfords, GE (light blue) = Goat Mountain early flight, GL (dark blue) = Goat Mountain late flight, ME (light green) = Mendocino Pass early flight, ML (dark green) = Mendocino Pass late flight, OR (purple) = Oregon.

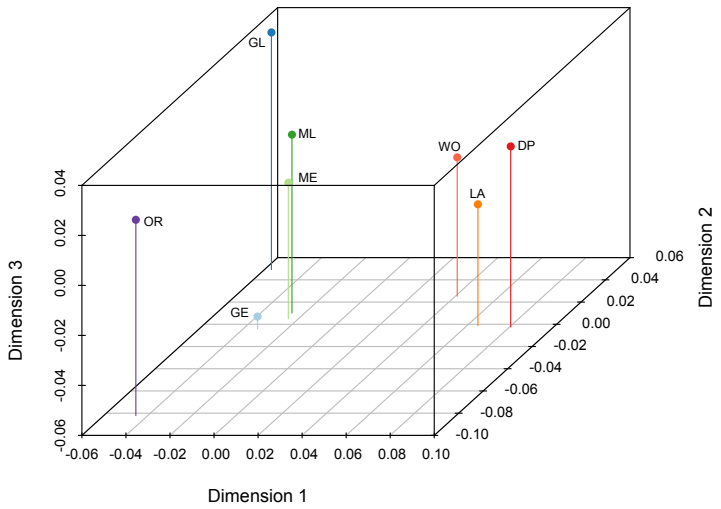

Figure 4: Non-metric Multi-dimensional Scaling (NMDS) graph of pairwise G_ST estimates among populations of *N. menapia*, showing 3 dimensions; DP (red) = Donner Pass, LA (orange) = Lang, WO (light red) = Woodfords, GE (light blue) = Goat Mountain early flight, GL (dark blue) = Goat Mountain late flight, ME (light green) = Mendocino Pass early flight, ML (dark green) = Mendocino Pass late flight, OR (purple) = Oregon.

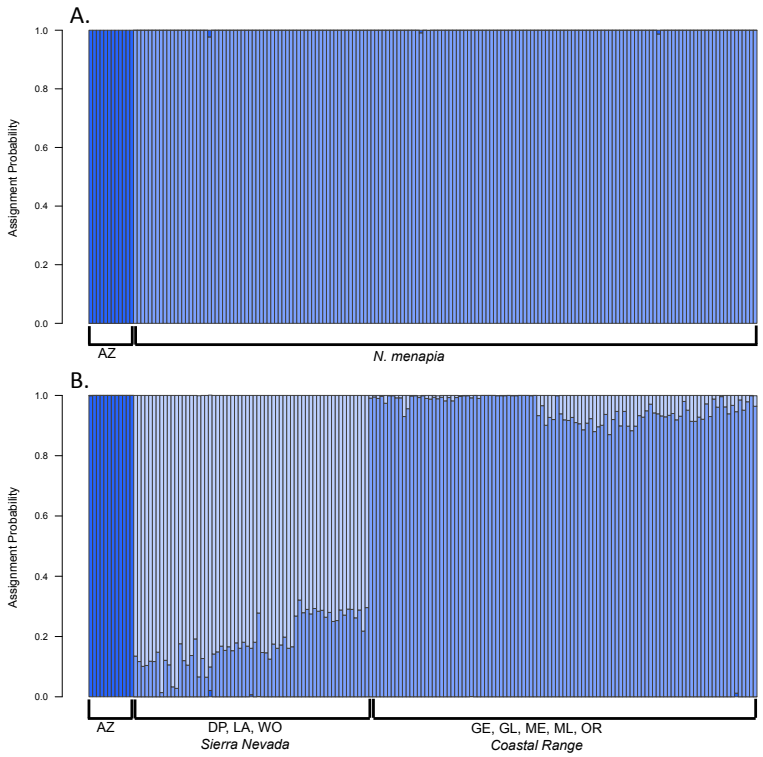

Figure 5: A: STRUCTURE assignment plot for K=2, includes all populations samples (*N. terloottii* and *N. menapia*); dark blue = AZ (*N. terloottii*), medium blue = all *N. menapia* populations. B: STRUCTURE assignment plot for K=3, includes all populations samples (*N. terloottii* and *N. menapia*), dark blue = AZ, light blue = Sierra Nevada *N. menapia*, medium blue = Coast Range *N. menpia*. AZ = *N. terloottii*, DP= Donner Pass, GE = Goat Mountain early flight, GL = Goat Mountain late flight, LA = Lang, ME = Mendocino Pass early flight, ML = Mendocino Pass late flight, OR = Oregon, WO = Woodfords

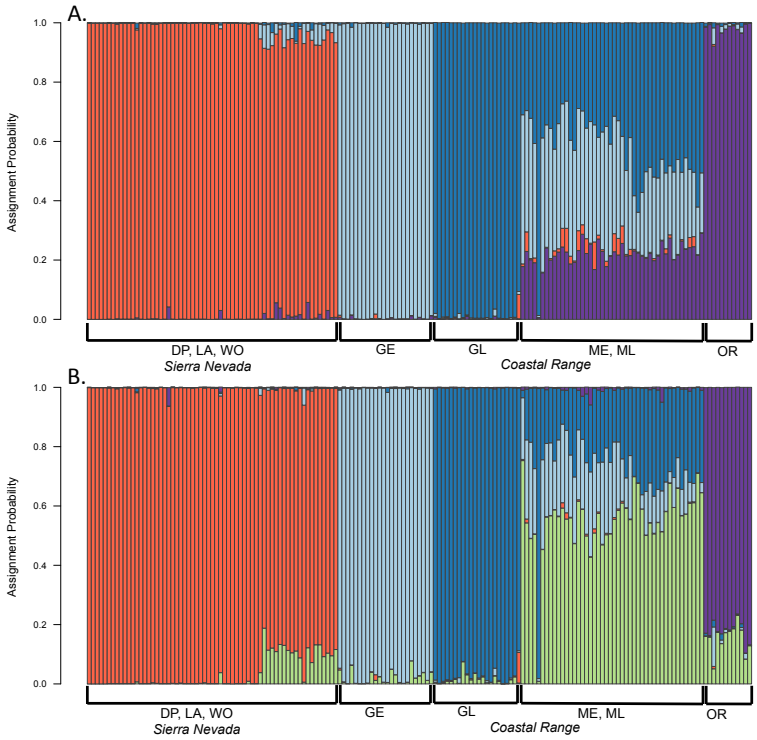

Figure 6: STRUCTURE assignment plots for all 8 *N. menapia* population samples. A: Assignment probabilities from STRUCTURE for K=4, orange = Sierra Nevada populations, light blue= GE, dark blue = GL, purple = Oregon. B: Assignment probabilities for K= 5, orange = Sierra Nevada populations, light blue = GE, dark blue = GL, green = Mendocino Pass, purple = Oregon. DP= Donner Pass, LA = Lang, WO = Woodfords, GE = Goat Mountain early flight, GL = Goat Mountain late flight, ME = Mendocino Pass early flight, ML = Mendocino Pass late flight, OR = Oregon.

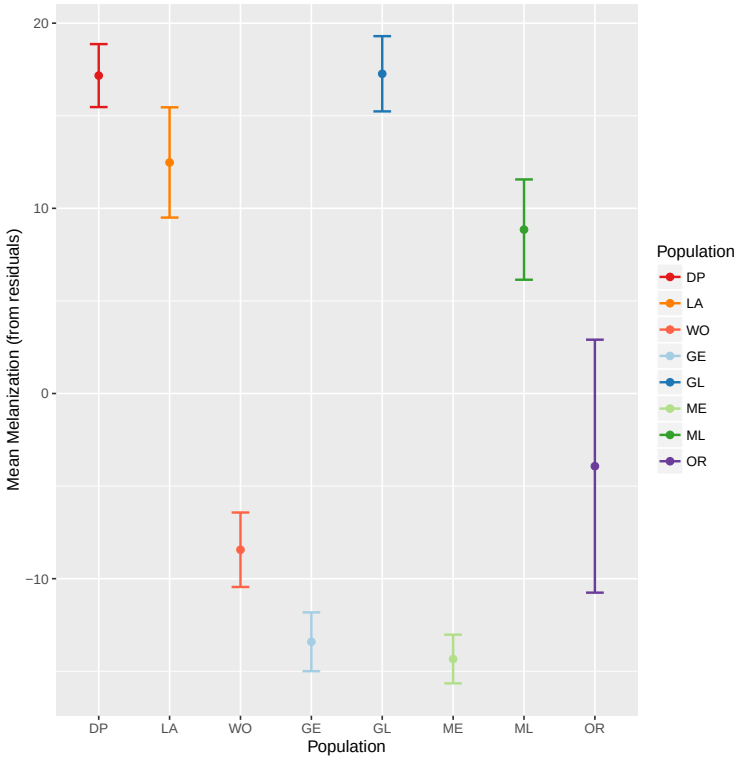

Figure 7: Boxplots of melanization level for populations of *Neophasia menapia*. DP = Donner Pass, GE = Goat Mountain early flight, GL = goat Mountain late flight, LA = Lang, ME = Mendocino Pass early flight, ML = Mendocino Pass late flight, OR = Oregon, WO = Woodfords.

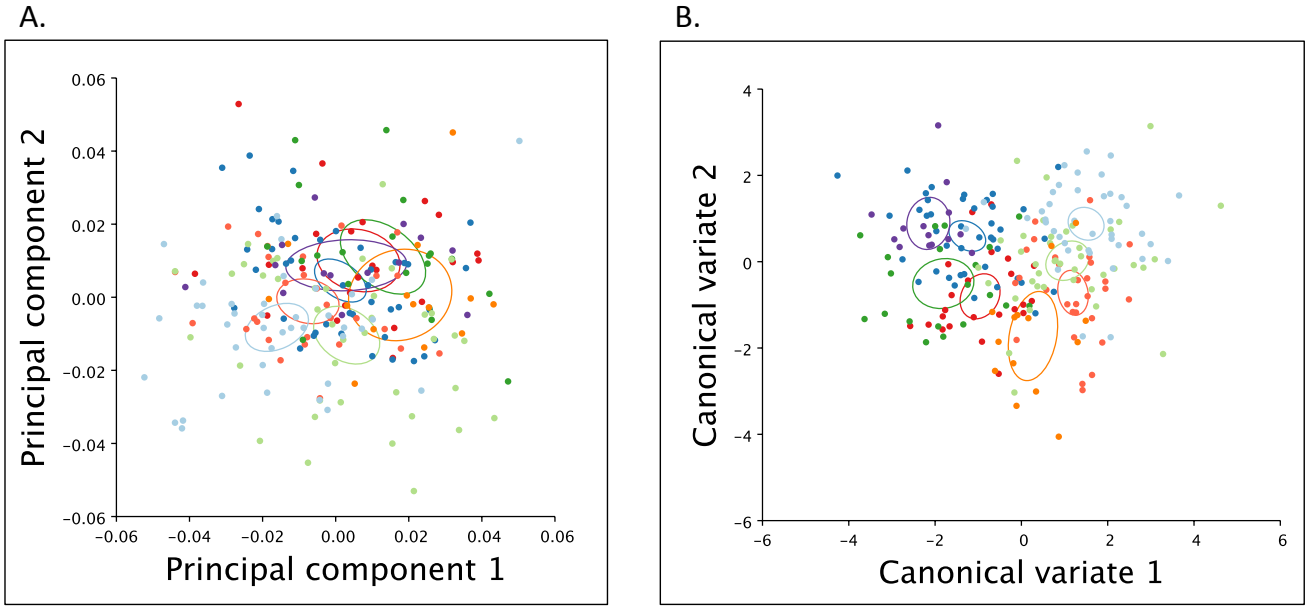

Figure 8: A: PCA of *N. menapia* wing landmarks, 95% confidences ellipses around the mean for each population. B: CVA of *N. menapia* wing landmarks, 95% confidence ellipses around the mean for each population. *N. menapia* samples from; DP (red) = Donner Pass, LA (orange) = Lang, WO (light red) = Woodfords, GE (light blue) = Goat Mountain early flight, GL (dark blue) = Goat Mountain late flight, ME (light green) = Mendocino Pass early flight, ML (dark green) = Mendocino Pass late flight, OR (purple) = Oregon.

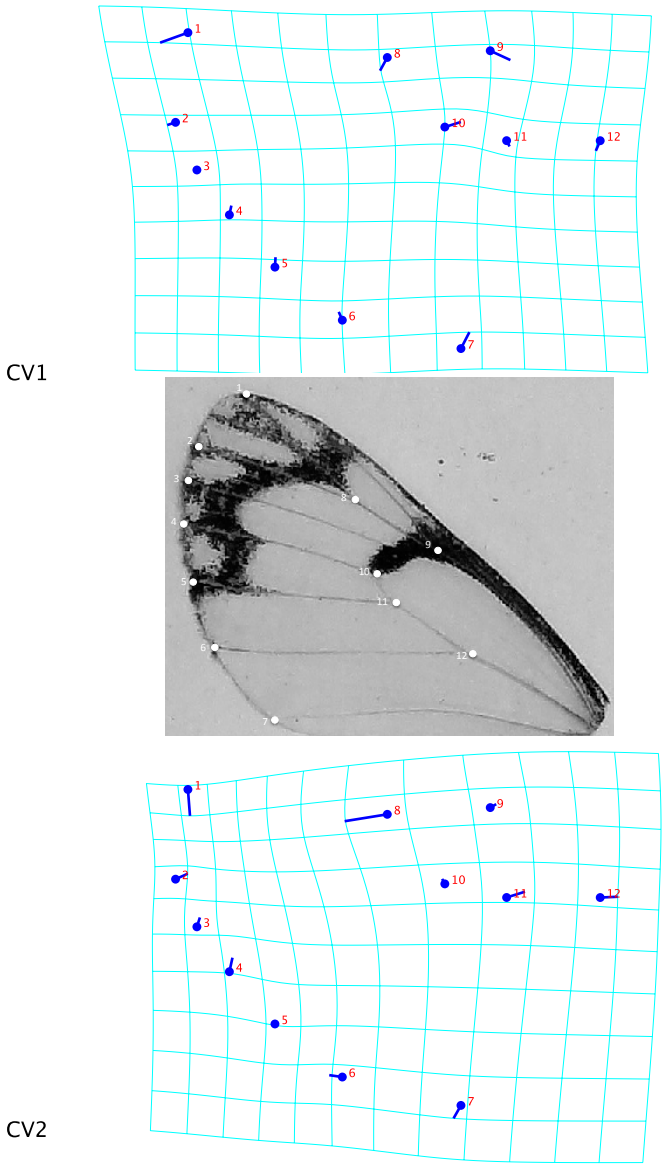

Figure 9: Transformation grid for landmarks from CV1 (top) and CV2 (lower).