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WING SCALE MORPHOLOGY OF A SEXUALLY DIMORPHIC COLOR PATTERN ELEMENT IN THE BUTTERFLY CO-MIMICS *HELICONIUS MELPOMENE* AND *HELICONIUS ERATO*

by

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ABSTRACT:

Heliconius, a Neotropical genus of butterfly, has served as a model organism for the study of mimicry since the 19th century. Despite the extensive research conducted on this organism, very little is known about sexually dimorphic elements of their wing patterns. In this study, I examined the characteristics of the overlap band, an area of shiny, silvery/brown scales on the posterior ventral forewing and the anterior dorsal hindwing. Males and females are sexually dimorphic in regards to the length and breadth of the area covered by these scales. Males also have sex-specific, pheromone-releasing scales known as androconia within this region. In order to determine if sexual dimorphism existed on the level of the wing scales, beyond the presence or absence of androconia, I extensively sampled the overlap band of four specimens: a male and female from each of the co-mimics *Heliconius melpomene* and *Heliconius erato*. By analyzing the distributions of classes of scale shapes, I found strong trends in how scale shape changes across the overlap band. These trends, with variations, are present in both species, male and female, which raise questions about the control and organization of wing scale development in two relatively distant members of the genus.

INTRODUCTION:

Since the beginning of the study of natural history, moths and butterflies have held a strong fascination for those who wish to unravel the secrets of the natural world. The startling colors and bold patterns that in a wing beat turn to bark or dead leaves, attract both scientists and the general public. Upon the delicate, fluttering wings resides a visible connection between predator and prey, male and female, gene and cell, that provides an opportunity to see the forces that drive biology turned into mosaics, mirrors and codes. Some of the first work in evolution was conducted on the multitudinous patterns of Lepidoptera. Henry Walter Bates, the naturalist namesake of Batesian mimicry, discovered a ring of poisonous models copied by harmless mimics in the Amazon that was an early triumph of the Darwinian evolutionary narrative (Brown, 1981). Moths and butterflies also played an essential role in the work of Fritz Müller, who proposed the opposing theory of Müllerian mimicry, where two toxic organisms copy each other. Other biologists, such as Poulton, Punnett, Fisher and Goldschmidt used Lepidoptera to craft some of our foundational ideas about evolution.

Upon the surface of a moth or butterfly's wing, tiny, individually colored scales serve to create the innumerable patterns seen across the order Lepidoptera. Each scale is the result of a developmental process during metamorphosis that shapes and colors a single living cell into a chitinous tomb. Together, these

multitudes of dead cells form the color patterns of the wing, each one much like a pixel on a computer screen.

These patterns play several important roles in the life of the insect. They can be used for camouflage, to warn of distasteful or poisonous properties, to mimic other Lepidoptera or other insects, to attract mates or to frighten off attackers. As such, these patterns serve as a confluence where ecology, physiology, behavior, genetics and development converge in a visible and striking manner (Joron *et al*., 2006a).

From the dawn of Darwinian biology, the genus *Heliconius* has served as a kaleidoscope of evolutionary stories, and has captivated scientists since the 19th century. Species of these butterflies, which are members of the Nymphalidae family, inhabit tropical South America, Central America and some southern regions of the United States. Populations of *Heliconius* provide a considerable array of possibilities for biological study, but perhaps the most obvious is their brilliant black, red, white and yellow color patterns. Beyond the beauty of their bands and stripes, these designs serve two vital functions in the life of the butterfly: aposematism and mimicry. As larvae, *Heliconius* feed on toxic passion flowers, which allows them to synthesize poisonous compounds that make them distasteful to predators. This is augmented in adulthood by feeding on pollen of poisonous tropical cucumbers. *Heliconius* indicate their unpalatability to predators with the brilliant colors and bold patterns on their wings. In an evolutionary phenomenon known as Müllerian mimicry, co-occurring species of

poisonous *Heliconius* closely mimic each other's patterns. Predators learn to avoid a certain pattern of colors and shapes, and all the butterflies that fly it share the costs and benefits of this tutelage (Langham, 2004). This shared protection has been demonstrated by experiments in which *Heliconius* models with altered patterns were attacked by predators much more often than artificial butterflies bearing the typical warning coloration for that region (Langham, 2004). *Heliconius* are not the only ones in on the secret, however. Other species of butterflies, and even moths, serve as mimics and models, creating a complex web of Müllerian and Batesian mimicry that stretches over several families of Lepidoptera.

Beyond their color patterns, *Heliconius* possess several other characteristics and adaptations that make them an important and fascinating genus to study. As mentioned earlier, adult heliconiid butterflies participate in an unusual feeding mechanism. The vast majority of Lepidoptera use their straw-like proboscises to sip sugary nectar from flowers. *Heliconius* often feed instead on the protein-rich, poisonous pollen of several genera of cucurbits. Prodigious amounts of saliva glue the pollen grains to the proboscis of the butterfly, and also begin to digest the pollen with the aid of physical manipulation. The resulting amino acid rich fluid is then absorbed by the proboscis (Beltran *et al*., 2006; Gilbert, 1972; Krenn and Penz, 1998). This unique feeding mechanism requires no obvious structural modifications in the *Heliconius* mouthparts, and seems mainly to be an inherited behavioral adaptation (Krenn and Penz, 1998). The diets

of other butterflies consist mainly of the sucrose acquired from nectar, with only trace amounts of protein. This lack of amino acids, some of which cannot be synthesized and must be obtained from an outside source, limits the life span of the insect to a few weeks (Gilbert, 1972). With the abundant amino acids digested from pollen, however, *Heliconius* can live for up to six months, and also boost egg and spermatophore production and provide supplementary building blocks for the synthesis of the cyanogenic glycosides that make them unpalatable (Brown, 1981). Females of some *Heliconius* species take advantage of their long life spans by mating multiple times, digesting the spermatophores of their partners. They use these nuptial gifts to build up reserves of protein and poisonous compounds for egg laying, and usually only use one male's sperm to fertilize eggs (Cardoso, Roper and Gilbert, 2009).

Some *Heliconius* take a much different approach to reproduction. The genus is divided into two nearly equal-sized clades by the presence or absence of a unique mating strategy. One group mates as adults like most nymphalid butterflies. The other clade, however, has a different strategy known as pupal-mating. Large males from this group search for a female pupa that is close to emerging, a state that she announces by the release of pheromones (Estrada *et al*., 2011). Once the male has found his potential mate, he guards her from all interlopers (Estrada and Gilbert, 2010). As the female begins to eclose, the male will either insert his abdomen into the chrysalis to mate with her, or he will copulate with her immediately after she emerges. Some males, often smaller in

size, participate in patrolling for females like adult-maters, rather than guarding pupae (Mendoza-Cuenca and Macías-Ordóñez, 2009).

This odd strategy poses several intriguing questions. First, the cuticular structures of the female, including the genitalia, usually do not fully harden until after eclosion. So, how does the male mate with the female without damage? Precocious hardening of the female genitalia would seem to be a useful modification in this clade, but I have seen no papers that describe it. Perhaps there is no adaptation for early cuticular hardening, and the males use this as a method to prevent further mating. If her genitalia are damaged during pupal-mating, future matings as an adult would be futile. Indeed, females of pupal-mating species rarely mate again in their lifetime (Cardoso, Roper and Gilbert, 2009). Why undergo such an odd mating behavior in the first place? This mating tactic has not been observed in butterflies outside of this clade of *Heliconius*, although it often occurs in other insects. (Beltran *et al*., 2006). This strategy seems to negate female choice, though it has been suggested that it could serve as an indirect form, where the female benefits by receiving sperm only from males that can successfully guard the pupa, especially if these traits are heritable and passed down to her sons (Estrada *et al*., 2009; Mendoza-Cuenca and Macías-Ordóñez, 2009). **[**Pupal-mating females rarely mate more than once, but still live an inordinately long time for an insect (Walters *et al*., 2012). Perhaps her long life means she can be extremely particular about where she lays her eggs, allowing her to give her offspring a better chance of survival. Or perhaps, it shows that

pupal mating is a derived trait, and their long lives are carried over from the adult-mating clade.

A fascinating and poorly understood aspect of pupal- versus adult-mating is that when two *Heliconius* species mimic each other, it is typical that one member will be a pupal-mater, and one an adult-mater. It has been suggested that their widely divergent mating strategies allow for mimics to live sympatrically in an area with minimal species recognition errors (Beltran *et al*., 2006). Pairs of mimicking species often have multiple subspecies with distinctive patterns that vary from one geographic location to the next. In each region, the co-mimics still have the same pattern as their partner, resulting in convoluted web of species, subspecies and mimics that can be difficult to sort into their component parts. For example, two of the best studied mimics, *Heliconius melpomene* and *Heliconius erato*, have over 20 different wing pattern races scattered throughout the Neotropics (Hines *et al*., 2011). Inferences from genetic and population history of this co-mimic pair indicate that the pupal-mater, *H. erato*, initiated the patterns seen in each geographic location, and was subsequently copied by the adult-mater, *H. melpomene* (Flanagan *et al*., 2004).

Mimicry plays an immensely important role in the evolution and ecology of *Heliconius*, and this significant phenotypic trait is reflected in the genome. Many organisms have evolved tightly linked genetic constructs, known as supergenes, that involve several loci that are inherited as a single unit (Jones *et al*., 2012). In many *Heliconius*, alleles of a loci control the phenotype of the color

pattern displayed on the wings. In many species, even distantly related ones such as *H. melpomene* and *H. erato,* these loci have been found to homologous and fairly conserved (Joron *et al*., 2006b). Amazingly, the diverse arrays of color patterns seen throughout the genus are actually, for the most part, controlled by only a few loci. *Heliconius numata* takes this one step farther, and has one supergene that controls the majority of its wing pattern phenotype, which is homologous to the major effect loci of *H. melpomene* and *H. erato* (Jones *et al*., 2012). The origin of this remarkable mechanism is believed to have arisen from the importance of the mimetic patterns in *Heliconius* ecology. If only a few, tightly linked loci control most of the elements of the color pattern, then there is less of a chance that recombination or mating with different color morphs will result in a non-mimetic, and therefore inadaptive, pattern (Jones *et al*., 2012). It also means that an allele switch or mutation in the supergene can quickly create a new phenotype, which might allow the pairs of *Heliconius* species to track each other where they co-occur (Hines *et al*., 2011).

The patterning of any butterfly wing not only prompts fascinating questions of ecology and evolution, but also the cellular biology of cytoskeleton and the interactions between the cells that will become the scale and its associated structures. Each scale originates from a single, living epithelial cell within the developing wing of the pupal butterfly. During metamorphosis, the scale cell projects an extension above the rest of the cells of the developing wing surface (Kristensen and Simonsen, 2003). The cell then undergoes a series of complex

cytoskeletal gymnastics to form it into the final shape of the scale. A day or so before eclosion, the scale cell synthesizes the pigment that will later give the dead scale its color (Nijhout, 1991). In order to become a mature scale, the precursor cells die, leaving their complex, highly structured husks attached to the wing membrane (Nijhout, 1991).

All scales have a general base plan, though the form of a scale can vary widely depending on its color, function and placement on the wing. Most scales are a thin, squashed balloon, with the two faces formed of chitinous cuticle and tanned proteins, sandwiching a layer of air in between (Kristensen and Simonsen, 2003). The cuticle forms many different structures on the scale, known collectively as the ultrastructure, such as the longitudinal ridges found on the surface of the scale, the inner trabeculae that connect the two sides, and the architecture that can produce physical, as opposed to pigment-based, colors (Kristensen and Simonsen, 2003). In *Heliconius* the color of the scale is determined by chemical pigments and has been found to be closely linked to different types of ultrastructure (Gilbert *et al*., 1988). The main body, or "blade," of the scale is supported and attached to the wing membrane by a thin basal stalk, the pedicel (Kristensen and Simonsen, 2003). The pedicel inserts into another epithelially derived structure known as a socket cell, which protrudes from the wing membrane at a sharp angle, so that the scales lie nearly parallel to the surface (Downey and Allyn, 1975).

In the higher groups of Lepidoptera, of which *Heliconius* is a part, the wing scales are arranged across the wing in nearly regular, transverse lines (Kristensen and Simonsen, 2003; Aymone, Valente and de Araújo, 2013). The scales lie in rows that are roughly parallel to the distal edge of the wing, with a shingled arrangement so that each scale overlaps the base of the one distal to it (Nijhout, 1991). In most Lepidoptera, there is not one, but two layers of scales on the wing; a bottom layer of smaller ground scales, and an upper layer of large, often more brightly colored cover scales (Kristensen and Simonsen, 2003; Nijhout, 1991). During development, the size of the scale cells (which is determined by ploidy) alternates regularly along each row, with the larger cells giving rise to cover scales and the smaller to ground (Nijhout, 1991). Some areas of the wing can have simpler or more complex layering, which is believed to affect the aerodynamics of the membrane (Kristensen and Simonsen, 2003). In my specimens, I found that there is a single layer of mainly acute scales along the inner and costal margins of the fore and hindwings respectively.

Scales serve as units of color, and therefore pattern, but they can provide other functions in the life of the insect. Dark colored scales on the wing can aid in thermoregulation by absorbing solar radiation, and scales on the body can provide insulation and help prevent heat loss (Kristensen and Simonsen, 2003). In many families of Lepidoptera, some scales have become highly differentiated, often with frilled, hairy or elongated margins. These scales, known as androconia, often occur in “pouches” on the body, legs or wings of males. While research into their

physiology and development is sparse, observation and chemical analysis indicate that androconia distribute and perhaps produce or store, male pheromones that are released during courtship (Andersson *et al*., 2007). Most androconia seem to disseminate pheromones through evaporation, which would explain the observation that they are quite often found in "tents" of other scales or folds in the wing. Such protective structures would prevent the loss of pheromones when not actively engaged in courtship (Kristensen and Simonsen, 2003). In the areas of the wings I studied, androconia made up an important component of the male vestiture. They seem to be overlaid by cover scales, but it might be reasonable that wing movements during courtship could expose them.

The evolution of scales allows the Lepidoptera to develop different patterns on the dorsal and ventral sides of their wings, since pigment is compartmentalized into individual units (scales), rather than embedded directly into the transparent membrane of the wing as in other insects (Kristensen and Simonsen, 2003). The possibilities for butterfly wing patterns, then, are vast, even within a species. *Heliconius* in general, and the two species I studied in particular, *H. melpomene* and *H. erato*, show a spectacular array of color morphs. Since close mimicry plays such a strong role in their ecology, species of *Heliconius* rarely exhibit overt sexual dimorphism in their color patterns (Brown, 1981). While some differences in size and morphology are tied to sex, the overall wing pattern remains largely unchanged. In *H. melpomene* and *H. erato* (as well as other *Heliconius*), however, one element of the color pattern differs noticeably

between males and females. This pattern element, often gold or grey in color, occurs on the posterior ventral portion of the forewing, and the anterior dorsal area of the hind wing. At normal rest or in flight, these two areas overlap, and are therefore hidden from sight. Different phenotypes between males and females in this "overlap band" can be easily recognized. In males, grey or gold coloration almost completely fills the two most anterior wing cells of the dorsal surface of the hindwing, and the two most posterior forewing cells on the ventral surface. Females, however, exhibit an overlap band that is more restricted, and is both shorter and narrower compared to the dimensions of the wing cells.

Despite the fact that the overlap band has been known to naturalists since the early 1900's (Riffarth, 1901), very little contemporary work has been conducted on it. In the compiled, systematic treatise on butterflies and moths The Macrolepidopetera of the World, Adalbert Seitz (1924) describes the overlap band as a "friction surface," with a glossy texture and lacking tufts or hair pencils that related groups such as the tribe Ithomiini possess. The characteristics of the overlap band led to a discovery vital to our understanding of the complex natural history of the *Heliconius*. Heinrich Riffarth, a German naturalist in the early 20th century, noticed that the genus could be divided into two groups by a trait found on the underside of the male forewing (Kaye, 1907). In Group 1, officially known as the Opisogymni and later to be determined as the adult-mating group, the grey, reflective scales found along the inner margin of the forewing (that is, the overlap band) extend anteriorly until they touch the "median nervure," which is known

today as the second cubital vein, or CuA2 (Kaye, 1907; Eltringham, 1916). In the other group, the Opisorhypari, or pupal-maters, these shiny scales became duller and darker before they reached CuA2, leaving a thin strip between the vein and the overlap band (Kaye, 1907; Eltringham, 1916).

Using this characteristic, Riffarth not only divided the genus into two groups, but also found that, in many instances, butterflies that appeared to belong to one species were actually separate species with extremely similar patterns (Riffarth, 1901). Riffarth's division of *Heliconius* into two groups stands largely unchallenged to this day, despite a few name changes and the reassignment of some species to subspecies status. His two groups, based on the morphological features of the overlap band, were later discovered to be the pupal-mating (Opisorhypari) and the adult-mating (Opisogymni) clades. A further examination of Riffarth's classification by the English naturalist Walter J Kaye lead to an additional important realization. He noticed that species with very similar patterns often seemed to not only occur in the same geographic location, but also to occur in pairs, with one from each group (Kaye, 1907). This observation also stands, and has been corroborated with DNA sequencing, that co-mimic pairs quite often consist of one pupal-mater and one adult mater.

Even though the overlap band is undeniably important in classification, at least, very little contemporary research has been conducted concerning its evolution and function. One recent study examined the ultrastructure of the scales found in the overlap band, and compared their findings to previous research on

Heliconius scales (Aymone, Valente and de Araújo, 2013). Their results indicate that the overlap band could serve as a useful tool for understanding how the development, pigmentation, and ultrastructure of the scales are connected (Aymone, Valente and de Araújo, 2013). The selective or developmental factors that shape the evolution of the overlap band remains elusive, but the concentration of androconia in this area must be involved. During courtship flights, the overlap band of the male is clearly visible, suggesting that it plays a role in mating (Klein and de Araújo, 2010). In one of the only studies describing *Heliconius* courtship, all successful matings were preceded by androconia exposition, where the male exposes the overlap band to the female (Klein and de Araújo, 2010). Where androconia have been studied in Lepidoptera, these pheromones serve to make the female more receptive to the male's advances, and increase his chances of successful copulation. Some species, such as the cabbage butterfly *Pieris napi*, lack control over both the timing and quantity of pheromone released, which seems to be passively dispersed by the act of flight (Andersson *et al*., 2007). Other moths and butterflies actively incorporate androconia in courtship. Males of the species *Hipparchia semele*, or the grayling butterfly, attempt to catch the females' antennae between their wings, bringing them into direct contact with the androconia and the pheromones they bear (Andersson *et al*., 2007). These pheromones may also serve as a means of species recognition at close quarters, which would be an asset in such systems as *Heliconius*, where several species may be flying the same pattern in one area.

Though research on the mechanics of *Heliconius* courtship and copulation is sparse, most species are believed to have androconia. In *H. melpomene* and *H. erato,* the androconia reside largely on the anterior, dorsal side of the hind wing, within the overlap band. The presence of these scales in both an adult-mater and a pupal-mater raises intriguing questions. If the androconia are indeed used to induce females to be more receptive to mating, what purpose do they serve a male that copulates with a pupa? The pupal female is presumably powerless to reject the advances of the male, even if she could detect his pheromones while within the chrysalis. It could be that pupal-maters simply have not lost this character yet since diverging from the adult-maters, or there could be some reason for retaining this trait. Some studies have shown that some species of pupal-maters, such as *Heliconius charithonia* and *H. erato,* adopt two mating strategies, depending on male size. The larger, more robust males engage in pupal-guarding and pupal-mating, while smaller males actively patrol for females that eclosed without copulation (Mendoza-Cuenca and Macías-Ordóñez, 2009; Klein and de Araújo, 2010). This dual mating strategy could explain the presence of androconia in pupal-maters. According to one study, the divergent male mating strategies could be at least partially genetically determined, with environmental components influencing which morph will develop (Mendoza-Cuenca and Macías-Ordóñez, 2009). Based on this interpretation, an interesting route that this research could follow would be to examine males of pupal-mating species, and see if any

difference can be detected between the androconia of guarding males and patrolling males.

The scales of the overlap band display several intriguing features beyond the presence of androconia. The proportions of different scale shapes change depending on their location within the wing cell. To the naked eye, this creates the appearance of regions of subtly shifting colors and reflectance. Under a dissecting microscope, the individual scales become clear, revealing strands of shiny, sharply pointed shapes that gradually meld into larger, duller forests of rounded or rectangular forms. The scales of the overlap band, for the most part, have a hue that is very distinct from the rest of the wing, ranging from silvery grey to creamy yellow to light tan. The morphology of the scales, when examined under a light microscope, also retains an "affinity" for the overlap band. While the shapes of the oblong scales do not usually differ greatly from those found in what I'm calling the main pattern elements (the rest of the wing, excluding the overlap band), they are often more rounded or irregular. The finger-like projections on the distal margin of the scale, if present, seem to be more curved, and less sharply pointed. Near the distal edge of the wing, where the main pattern element encroaches on the overlap band, some scales exhibit morphologies intermediate between the two types.

In males, the distinctive overlap band scales extend nearly the edge of the wing and completely fill two cells in the forewing and hind wing, sometimes bleeding over marginally into a third cell. Females display a much more restricted

overlap band, with often much duller yellow and grey scales that recede halfway across the wing and only fully fill the width of one cell. The rest of the area that would be taken up by the overlap band in males has dark brown scales that are distinct in color from the deep black of the main pattern elements. Between the two species I examined, *H. melpomene* and *H. erato*, the males seemed fairly similar in phenotypes. Surprisingly, I did not see Riffarth's feature in the male *H.melpomene*; the scales of the overlap band did not seem to extend all the way to the CuA2 vein. I have observed Riffarth's feature in other males of the same species, however. This is possibly due to the face that the male used in my study was a mongrel of *H. melpomene* subspecies, which may have affected the phenotypes of the overlap band. The two females, though both showed a shorter, narrower phenotype, were somewhat different. In the *H. melpomene*, the transition between the yellow/grey scales was abrupt, with almost no gradation. In the proximal half of both the 2A cell of the forewing and the C cell of the hindwing, the yellow/grey scales resembled the color and reflectance of a male, before suddenly turning into brown. The *H. erato* displays a much more gradual change, with the typical overlap band scales grading almost imperceptibly into light brown.

During my preliminary examination of the overlap band, these are the features that I noticed and caught my interest. Such noticeable sexual dimorphism in two species that rely heavily on mimicry, and which show little to no dimorphism in their color patterns, seemed a characteristic worth examining. The

lack of research on this topic in an extensively researched genus of butterflies further spurred my interest in attempting to understand its complexities. Though I have no doubt that almost any angle of approach would have yielded intriguing results, I decided to examine the trends in scale shape distribution I observed, mainly because it seemed bounded in ways that other questions were not. Conducting behavioral experiments to determine the overlap band's role in courtship, attempting to discover the genetic or developmental pathways connected to it, or carrying out chemical analysis on the androconia and pheromones all would have proved exceedingly difficult in Mount Holyoke's facilities. While I could have made observations on courtship and mating behavior at the Magic Wings Butterfly Conservatory, the logistics would have been difficult, and the conditions were not ideal. The samples I had access to had been dead for varying amounts of time, with different methods of storage, making chemical analysis unreliable at best. Individual scale color would be another important aspect to investigate, but I do not have any idea how I could have studied that. Often, two scales that seem identical in color could have two very different pigments creating their hue. Or, if the color is determined by structure, two different architectures could be the basis of very similar shades. Because of these complications, it can be difficult to piece together what is actually creating the color seen in a single scale. By considering all these factors, I decided to investigate the patterns of scale shape distribution, and see what I could deduce.

METHODS AND MATERIALS:

The bulk of my research, such as the description of trends in the frequencies of scale types in each wing cell, is set forth in the spirit of exploratory data analysis. I closely examined a male and a female from two species, one from the pupal-mating clade (*H. erato*) and one from the adult-mating clade (*H. melpomene*) (Figure 1). The male *H. melpomene* came from the Magic Wings Butterfly Conservatory in South Deerfield, MA, and was a mongrel resulting from many generations of interbreeding by several unknown subspecies. The rest of my specimens were collected in Trinidad by John Morrall, and acquired through eBay. My female representative of the *H. melpomene* species is a *H. melpomene flagrans*, and was captured at Morne Catherine, Trinidad on 9-27-2011. The pupal-mating specimens were *H. erato adana*, and were caught on 10-3-2012 at Point Goude, Trinidad.

Since I only sampled four specimens, statistical analyses on most of my data proved impossible. However, important differences do exist between both sexes and species. From evidence presented in the literature on *Heliconius* and my own observations, only the hind wings of males have androconia present. The overlap band provided the initial evidence for the two clades of the genus, which were later supported by fundamental behavioral differences in mating and DNA sequencing data. The differences between the groups are well supported by completely different sets of data, and were not questions I was attempting to

answer. The data from my four specimens is not sufficient for a statistical approach concerning how the scales of the overlap band differ on a species level, but they do provide the foundation that such a study could be based upon. Rather, my research focused on a detailed examination of a little studied area of the wing in a small number of samples in order to gain a better understanding of its characteristics.

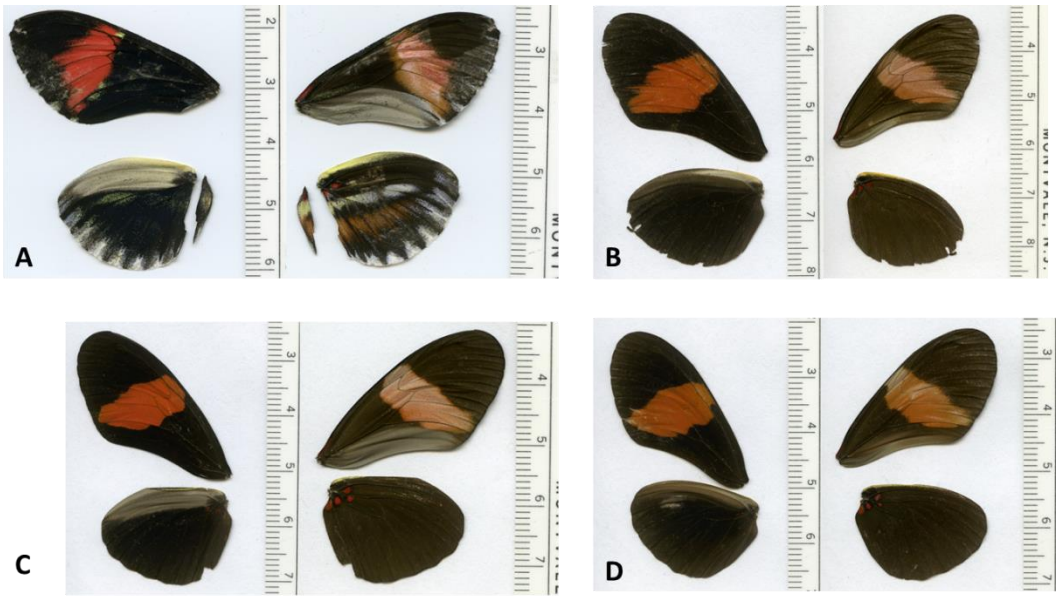

Figure 1.) The fore and hind wings of the specimens used to collect scales from the overlap band. **A** is a male *H. melpomene* subspecies hybrid from Magic Wings butterfly garden in South Deerfield, MA. **B** is a female *H. melpomene flagrans* collected from Morne Catherine, Trinidad (9-27-2011). **C** is a male *H. erato adana* collected from Point Goude, Trinidad (10-3-2012). **D** is a female *H. erato adana* collected from Point Goude, Trinidad (10-3-2012). Wings are not shown to scale with each other—ruler shown alongside is in increments of cm (numbered).

The overlap band consists of two wing cells of the forewing and two cells of the hind wing, and has different phenotypes in males and females of both species I examined (Figure 2). When discussing Lepidoptera, the term "cell" refers to the membrane between the wing veins. Conventionally, the wing cell is

named for the vein that is its anterior boundary. On the forewing, the cells of interest are the CuA2, named for the 2nd cubital vein, and the 2A, which takes its nomenclature from the 2nd anal vein (Figure 3). The 2A cell runs along the inner margin of the wing, and only has one vein bordering it (which, in this study, is referred to as a marginal cell), while the CuA2 cell is more anterior, and is nestled between the CuA2 and the 2A veins (and is called an interior cell in this paper). The two cells that make up the overlap band in the hindwing run along the anterior portion of the wing (Figure 4). The most anterior cell, the C or costal cell, is found along the costal margin of the wing and has only one vein bordering it, on the posterior side, which means it is a marginal cell. Below the C cell is the interior cell of the hindwing, the Sc+R1, which is located between the Sc+R1 vein (the subcostal and 1st radial vein, which fuse during development) and the Rs, or radial sector vein.

In order to examine how the morphology of the scales differs across the overlap band, I extensively sampled the wing in this area. The method I used to remove the scales, which I adapted from a technique developed by April Dinwiddie, a graduate student at Yale University, involved gently brushing against the wing cuticle with a minuten pin, which is a 12mm long, 0.20mm wide metal needle. In order to manipulate such a tiny piece of wire, I inserted it into a small wooden dowel, approximately 2mm in diameter, and glued the inserted end for stability. I bent the minuten pin near the tip, so that the last 1-2mm were

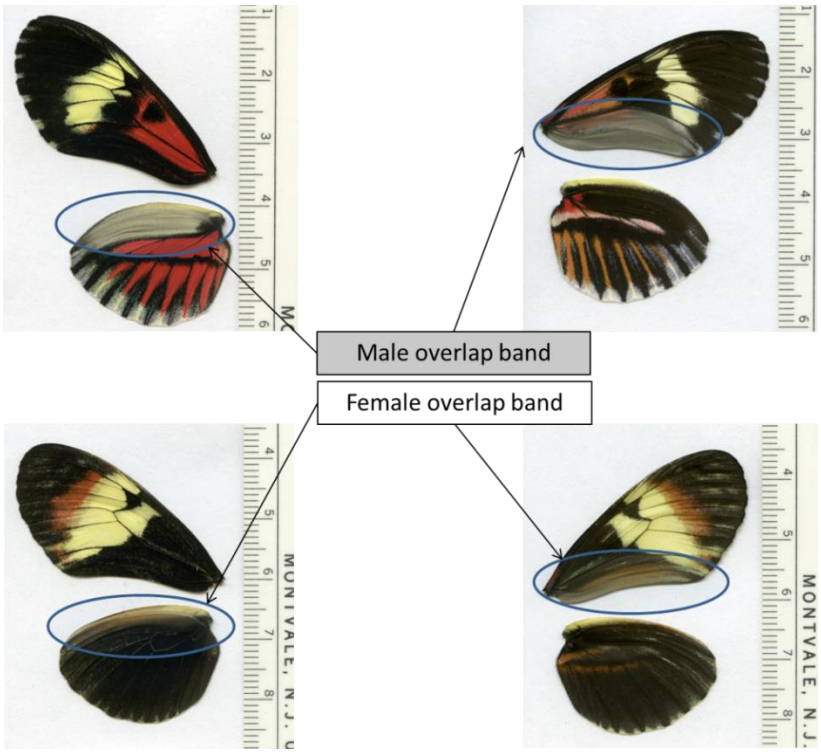

Figure 2.) Males and females of the species *H. melpomene* and *H. erato* have a sexually dimorphic pattern element called the overlap band. It occurs on the dorsal anterior hind wing and the ventral posterior forewing in both sexes. It is much more strikingly colored in males, and extends further across the wing both along a proximo-distal and anterior-posterior axis.

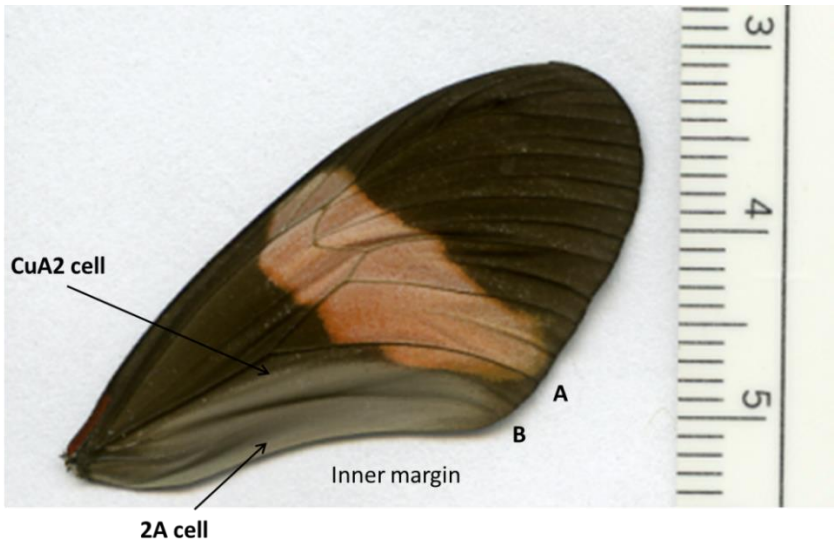

Figure 3.) Ventral forewing of a male *H. erato*, designating the cells of the overlap band. **A** indicates where the **CuA2** (the 2nd cubital) vein terminates at the distal margin of the wing. **B** indicates the termination of the **2A** (2nd anal) vein. The lower edge of the wing is known as the inner margin, and is the posterior edge of the 2A cell.

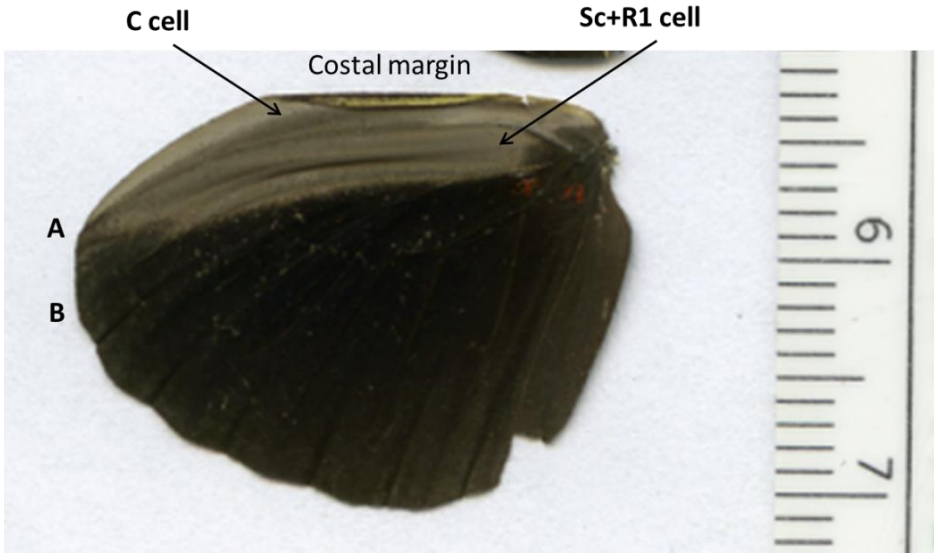

Figure 4.) Dorsal hindwing of a male *H. erato*, designating the wing cells of the overlap band. **A** indicates the termination of the **Sc+R1** (subcostal and 1st radial) vein at the distal margin of the wing. **B** shows where the **Rs** (radial sector) vein terminates. The upper edge of the hind wing is known as the costal margin, which is the anterior portion of the **C** (costal) cell.

angled, which provided a wider surface area for collecting the scales. The scales are so miniscule that static electricity causes them to adhere loosely to the wire of the minuten, and allows them to be transferred to a slide treated with a Poly-L lysine solution (0.02 grams Poly-L lysine, 396 mL water, 4 mL Tris pH 8). The chemically treated slides are slightly sticky, and hold the scales in place until a coverslip can be fixed over them.

To prepare the Poly-L lysine slides for my research, I used a method developed by April Dinwiddie. The slides have to be meticulously clean, since dust and dirt particles not only obscure the scales, but also make it difficult for the scales to be sufficiently flattened later in the process. Each slide was washed thoroughly in ethanol, and wiped clean with a Kimwipe (leaving slides to air-dry resulted in streaks that would impair visibility of the scales). Once the slides were

dry and free of ethanol, I placed them in a bath of Poly-L lysine for 10 minutes. After this period, I left them to dry on a covered rack, usually overnight, before storing them in a clean microscope slide box. As much as possible, I kept the slides out of the open air, since the Poly-L lysine adheres not only to the scales, but also dust and Kimwipe fibers.

Once the scales were removed from the wing surface, I carefully transferred them, on the minuten, to a Poly-L lysine slide. The scales "stick" fairly well to the minuten, though they can be easily disturbed by an errant breath or by bumping the dowel. With the aid of a dissecting microscope, I dragged the end of the minuten along the slide where I wanted to deposit the scales. Usually, depending on the number of scales attached or static electricity in the room that day, the scales easily adhered to the Poly-L lysine. The slide is sticky enough that the minuscule scales will not be dislodged by air currents in the room, but it is still possible to move them gently with the minuten. I found that spreading the scales out as much as possible, so that overlap is minimal, made counting and imaging them much easier. After all the scales were arranged on the slide, I placed a coverslip (like the slides, washed in ethanol) over them, and fixed it in place with fingernail polish. Since scales on the wing are curved, it is necessary to flatten them in order to be able to examine them under a light microscope. To do this, I used a system of weights applied to the coverslip, and left in place until the fingernail polish dried fully.

The methods I used to sample the wing allowed me to analyze the scales on two axes: proximal-distal, and anterior-posterior. The latter I examined on both a gross and fine scale. I sampled each wing cell in the overlap band at 9 proximal to distal sites, approximately 2mm apart. This means that each wing was sampled at 18 sites, and each specimen at a total of 36 sites. The sites began with 1 at the most proximal end of each cell, and ended with 9 at the distal margin of the wing. I divided each sampled area into anterior/posterior sections, taking scales from the more anterior (A) or posterior (P) region of the cell at each numbered site. This resulted in 9 sites that contained two sections each (1A/1P—9A/9P). I then subdivided these sections even farther into anterior (a) and posterior (p), so that there were four sampled areas for almost all sites (ex: 2Aa, 2Ap, 2Pa, 2Pp). 1 and 9 did not contain these subsections, as the width of the cell prevented further sampling. Refer to figures 5 and 6.

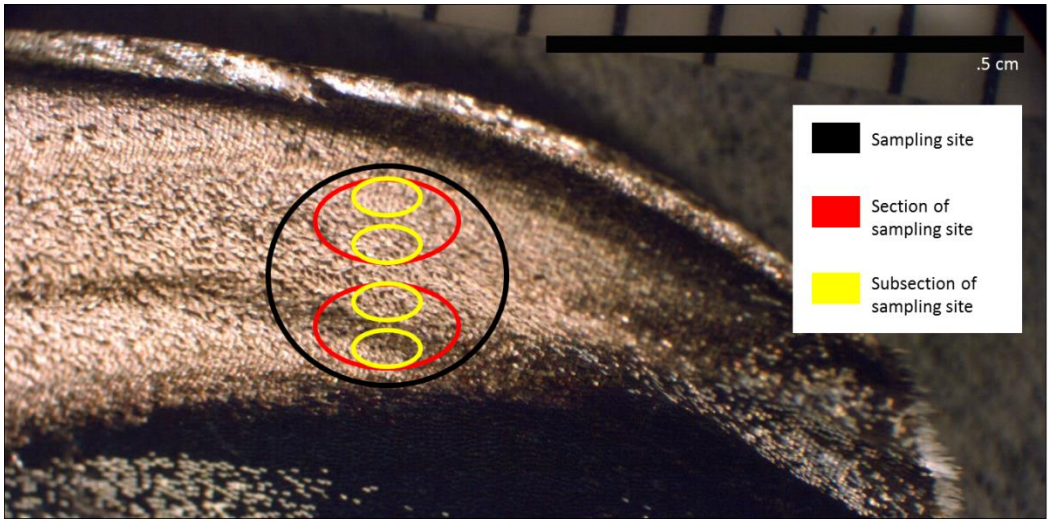

Figure 5.) Example of how sampling was done on the wing. The black circle indicates the sampling site (1-9), the two red circles indicate the anterior and posterior sections of the sampling sites (1A/1P—9A/9P), and the yellow circles show the anterior and posterior subsections (2Aa, 2Ap, 2Pa, 2Pp). Scale bar is 0.5 cm.

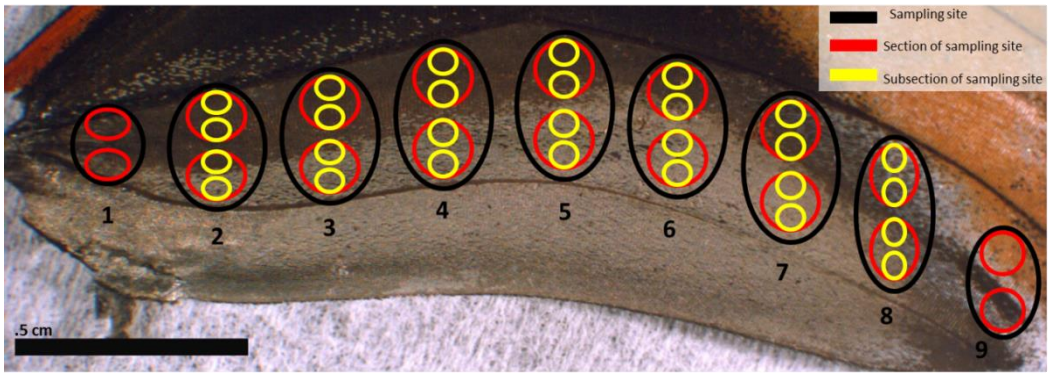

Figure 6.) Sampling sites across the wing. The most proximal and distal sites (1 and 9) lack the anterior/posterior subsections due to spatial constraints that made sampling in such a small area very difficult. Scale bar is 0.5 cm.

Under a dissecting microscope, it can easily be observed that the distribution of scale shapes change dramatically across the wing cells of the overlap band. In an attempt to analyze this phenomenon, I conducted visual scale counts of the sampled regions. Since accurately quantifying the number of scales directly on the wing can be quite difficult due to their shingled, overlapping organization, I used my prepared slides. Under a light microscope, at 40x magnification, I tallied the number of scales in each of 4-6 shape types in a single field of view. The average number of scales in the subsections of each sampling site ranged anywhere from 20-200, depending on the condition of the wing and how easily the scales detached from the cuticle. For each cell, the total number of scales ranged from 1000-3000, so that the total number of scales counted per specimen was between 4000-12,000. The most common shapes included acute/elliptical, oblong, oval and androconia, with the occasional appearance of linear or obovate scales (Figure 7). These shape categories were determined by comparison of the scales seen on my *Heliconius* samples and generalized scale

types described and used in the literature (Downey and Allyn, 1975). Often, the distal edge, or margin, of the scales within one shape category also appeared to change across the wing cell. In order to address this additional shift, I calculated the percentage of each shape category comprised of three different margin types: flat/round, convex/acute, and margins with tines (Figures 8 and 9).

Figure 7.) The four main scale types seen in the overlap band. **A** shows a general example of an acute scale, **B** shows an androconia from *H. melpomene*, **C** is an oval scale and **D** displays a general example of an oblong scale.

To visualize how the distribution of scale types shift across the wing along a proximo-distal axis, I calculated what fraction of each sampling site consisted of acute, oblong, oval, androconia, linear or obovate scales. From these data, I constructed bar graphs that showed the percentage of scale types versus sampling site, from the most proximal part of the wing to the most distal. I also used this technique to examine how the distribution of margin types changed within each scale type. To do this, I calculated the percentages of each margin type for each

scale type in each sampling site. In order to be able to see the distribution of scale types across the wing, I took the fraction of margin types from the percentage of that scale type in that sampling site.

These graphs show where trends occur along a proximo-distal axis. To illustrate changes along the anterior/posterior axis, I took the averages of the percentages of scale types in both the anterior and poster sections of the sampling sites. While this did not directly demonstrate how the distribution of scale types changed, it did give an idea of the general trend along an anterior/posterior axis. I constructed some scale percentage graphs using only the anterior or posterior sections, or even subsections, but the data sets in these samples were typically too small to show any consistent trends. The general organization of sampling sites from proximal to distal seemed to prevail, despite separating them into anterior and posterior sections. I also calculated the average percentage of scale and margin types across the wing cell to see if the results were consistent with the trends that I observed in the scale percentage graphs.

As a supplement to the more qualitative method of counting the scale types, I examined the statistical differences in the morphology of the scales, with the assistance of April Dinwiddie. I imaged 5-20 scales from each subsection of each sampling site, and Dinwiddie used 5 of these from each section to produce a more in-depth analysis of the shape variation. For a presentation and interpretation of the results, see Appendix A.

RESULTS:

Acute/oblong scales:

The two most common scale types seen in all of the specimens sampled are those with acute or oblong shapes. The defining characteristic of the acute type is that the distal end of the scale forms a sharp point. The body of the scale often resembles a triangle or an ellipse. While these scales can have more rounded margins, or even tines, they retain a fairly distinctive shape (Figure 8). The term oblong, on the other hand, describes a wider variety of scale shapes. I classified scales ranging from nearly perfect rectangles to half ovals as oblong scales. All the margin types can be found on oblong scales, though flat/rounded and tines are the most common (Figure 9).

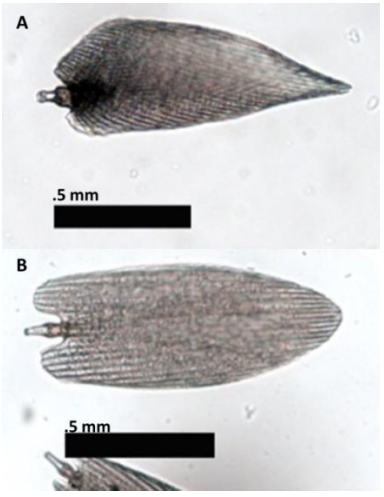

Figure 8.) The general margin types of the acute/elliptical scale type. **A** shows an convex/acute type margin, while **B** displays a flat/rounded margin.

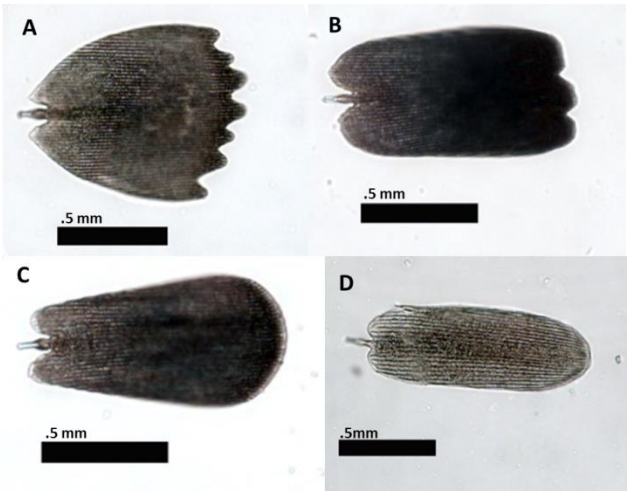

Figure 9.) Examples of the general margin types of oblong scales. **A** and **B** show oblong scales with tines. **C** displays a flat/rounded margin, and **D** shows a convex/acute margin.

Where acute and oblong scales are both present acute scales predominate in the proximal regions of the cell, and very few oblong scales occur. As you move towards the distal end of the wing, though, oblong scales become common and acute scales become rare. At the most distal sampling sites, almost all of the scales will be oblong, with few, if any, acute scales to be seen. This transition often happens in a gradient, so that the transition between oblong and acute scales is smooth. This relationship is seen most strongly in the marginal wing cells of the females in both sampled species, though it is present in the males as well. In both the 2A cell of the forewing and the C cell of the hindwing, acute scales make up nearly the entirety of the scales sampled in the proximal region, except for the very first sampling site (Figures 10 and 11). As the sampling sites move more distally, towards the edge of the wing, acute shapes give way to oblong, until 100% of scales sampled are oblong. The only disruption to this pattern comes from the very first sampling site. In all of my specimens, the 1A and 1P sites had mainly oblong scales, with only a few acute. In both males and females, this area is much darker than the rest of the overlap band, and resembles the main wing pattern in color (Figure 10). The scale shapes are unique, however, resembling neither the rest of the overlap band, nor the main pattern element scales (from what I have observed of it). They range from nearly circular to broadly obovate to asymmetrical rectangles with or without tines (Figure 11).

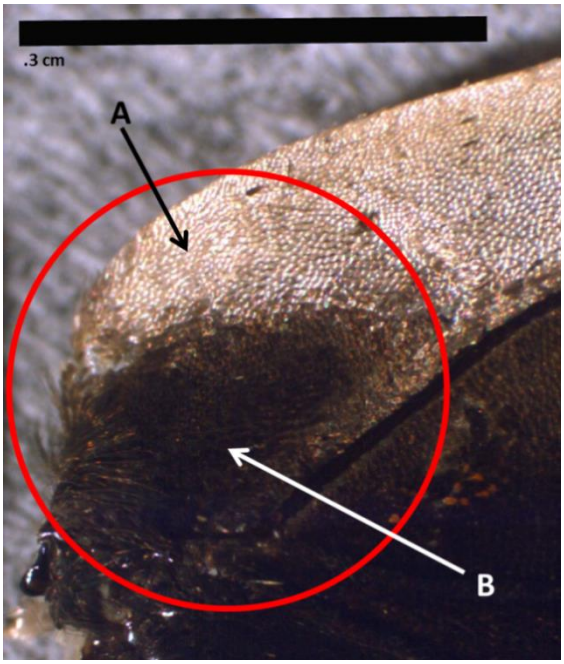

Figure 10.) Sampling site 1 of the C cell of the hindwing (of a female *H. melpomene*, though all specimens showed a similar trait). **A** indicates the anterior section, consisting almost entirely of acute scales. **B** shows the location of the posterior section, which is made up of oblong scales, often unlike those seen throughout the overlap band, or in the main pattern elements. Scale bar is 0.3 cm.

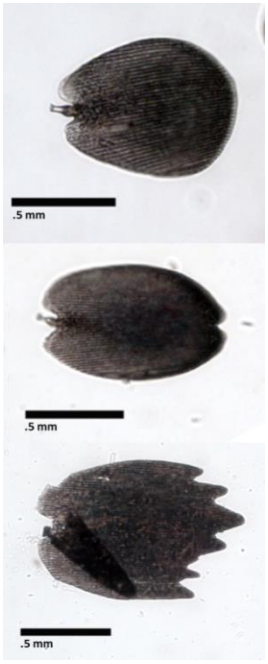

Figure 11.) Examples of the unique oblong scales found in the most proximal sampling site. For the most part, scales from this area resemble neither scales from the overlap band or main pattern element scales. Scale bar=0.5 mm.

This proximo-distal trend between acute and oblong scales is not as clear in males as it is in females. In the marginal C cell of the hind wing, the trend is obscured by the presence of many other scale types, such as androconia and oval, obovate and linear scales. Some evidence of such a trend is presented in the graphs created from my data, but it is not as strongly defined as in the females (Figure 12). Even in the male forewings, which lack androconia there is a weak indication that acute scales are more prevalent proximally and oblong distally, but not to the extent seen in the female wings.

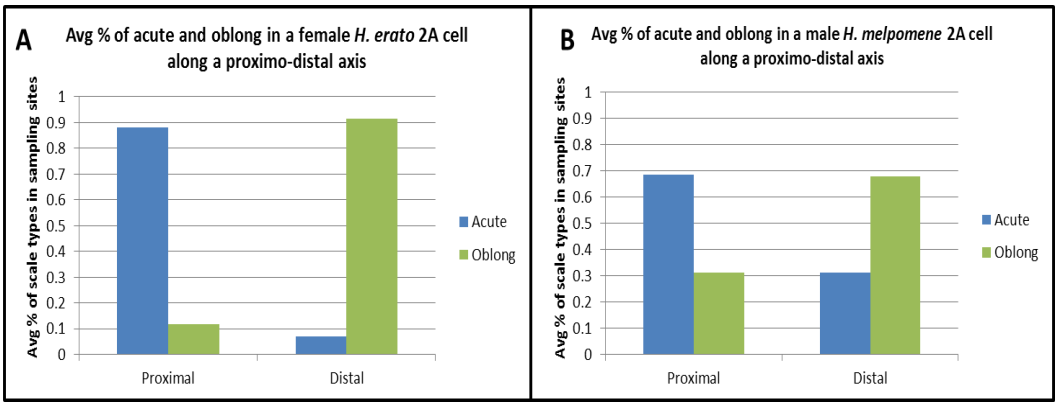

Figure 12.) Comparison of the average percentages of acute and oblong scale types in a female (**A**) and male (**B**) 2A cell. A female *H. erato* and a male *H. melpomene* are shown because they display the most obvious trend, but it is seen in both species. Total scale counts=1348 and 1797; average number of scales per sampling site=42 and 60 for the *H. erato* and the *H. melpomene* respectively.

In both males and females, this pattern is absent in the cells of the overlap band that are more interior, or bordered both anteriorly and posteriorly by wing veins. Here, the oblong scale type dominates almost exclusively. For males, the Sc+R1 cell of the hind wing also include androconia. The CuA2 cell on the forewings of the males, as well as all the interior cells (CuA2 and Sc+R1) of the females, consist of 80%-100% oblong scales. In the males, linear or oval scales are also present at low levels.

The marginal cells of the overlap band in the forewing and the hindwing display a shift in oblong and acute scale types along an anterior/posterior axis, as well as a proximo-distal one. This can be seen in the 2A cells of both the *H. melpomene* and *H. erato* males, where there are more acute scales in the posterior subsections along the wing margin, and where oblong scales are the dominant type in the anterior portions of the cell. Anteriorly, acute scales have an average percent of 31% and 29% (*melpomene* and *erato* respectively), and 68% and 61%

posteriorly, while oblong scales average at 63% and 70% in the anterior section of the cell, and 30% and 33% in the posterior (Figure 13).

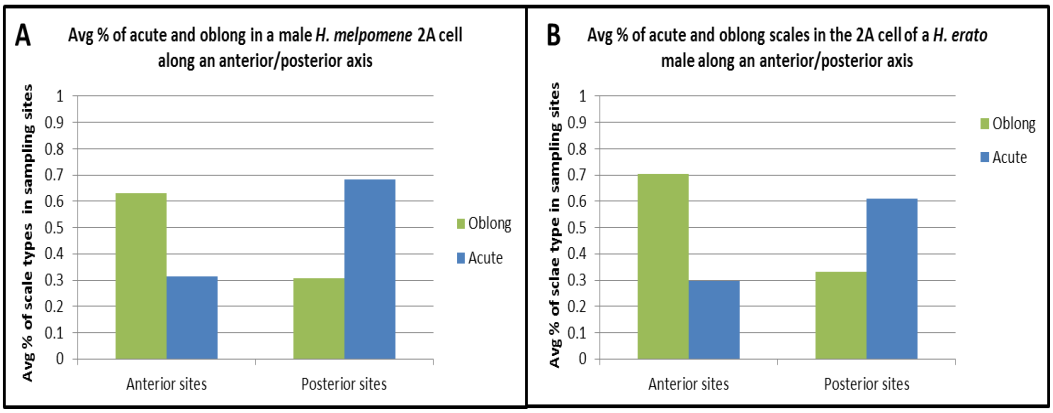

Figure 13.) Comparison of average acute and oblong scales in the 2A cells of a male *H. melpomene* and *H. erato*. Total scale count=1797 and 2714; average scales per sampling site=60 and 85 for *melpomene* and *erato* respectively.

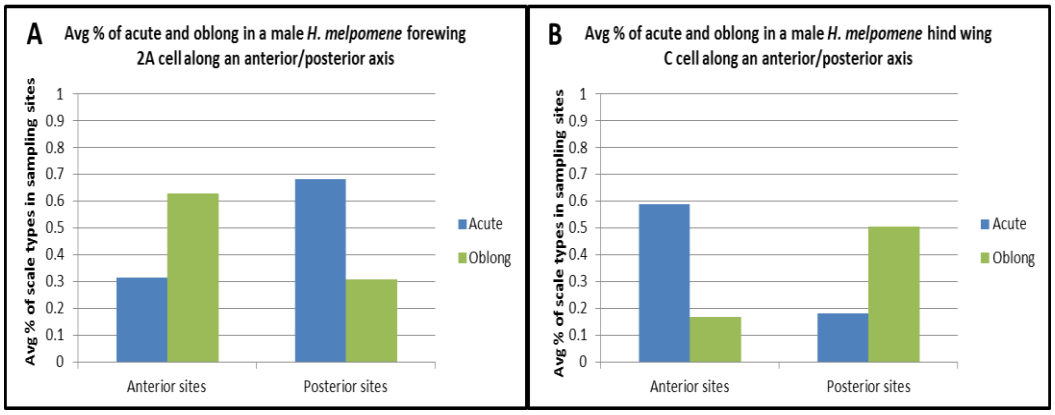

Figure 14.) Comparison of the distribution of acute and oblong scales in the marginal cells of the fore and hind wings. In the forewing , where the 2A cell is along the posterior edge of the wing, acute scales are more prevalent posteriorly. The opposite is displayed in the C cell of the hind wing, where the acute scales are more prevalent anteriorly. This supports the observation that acute scales are concentrated along the edges of the wing. Total scale count=1797 and 2497; average scales per sampling site=60 and 78 for the 2A and C cells respectively.

This trend changes orientation depending on the cell in question, however. In both species and both sexes, the acute scales are most heavily concentrated, and are the most acute, along the margin of the wing. This means that in the 2A cell of the

forewing, acute scales make up a higher percentage of the total scales in the posterior sampling sites, and a lower percentage in the anterior sites, or along the 2A vein. In the hind wing C cell, this relationship is reversed (Figure 14). The acute scales have a higher prevalence along the anterior of the cell, and taper off posteriorly.

The margins of oblong scales:

Oblong scales exhibit all three margin types in most cells, but the most prevalent are the flat/rounded margins or those with tines. For most of the cells, especially those along the lateral edge of the wing, no strong pattern of oblong margin type emerges. This seems to be especially true in the males, where the prevalence of acute scales is quite high until near the distal edge of the wing in both the 2A and C cells. The females also do not show much change in the distributions of margin type, though a high percentage of scale types are oblong as early as the 4^{th} and 5^{th} sampling sites. In both the C and 2A cells, the margins are a fairly even mixture of flat/round and tines, with a few convex/acute edges. There is no consistently dominant margin type in either cell, from the perspective of either sex or species—some have more flat/rounded margins, some have a greater percentage of tines, and some have nearly equal measures of both. There is no discernible shift in distribution of margin types in these edge cells, with the exception of the 2A of the *H. melpomene* female. In this specimen, the more proximal regions where oblong scales were present had a high percentage of

flat/rounded margins, which gave way to a high prevalence of margins with tines near the distal edge of the wing (Figure 15).

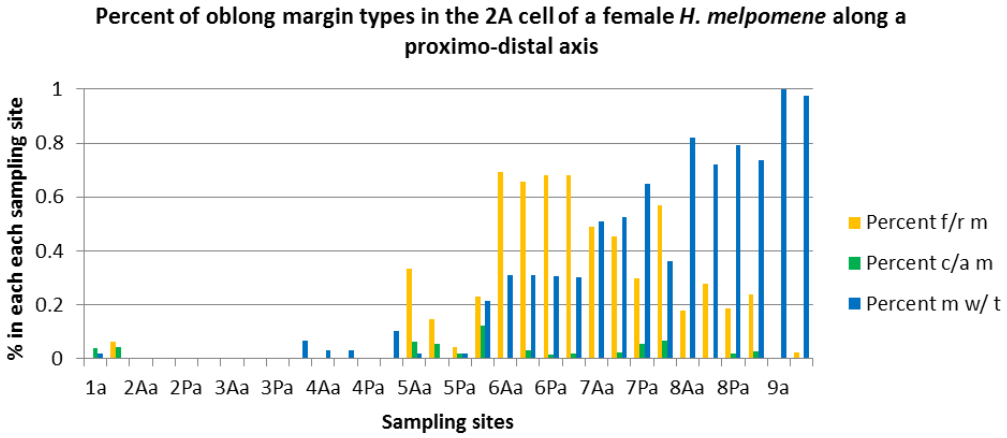

Figure 15.) Shifting distribution of oblong margin types in the 2A forewing cell of a female *H. melpomene*. In the sampling sites where oblong scales are present, flat/rounded margins (yellow) are more prevalent proximally, with tines with margins (blue) become more concentrated distally. Total scale count=1335; average scale count per sampling site=42.

Such a trend can also be seen, though to a less pronounced extent, within the more interior cells of the overlap band. In the CuA2 cells of both species and both sexes, the percentage of flat/rounded margins is much higher close to the body. Distally, however, these margins give way to those with tines, until almost all of the scales have two or more tines. This trend can be seen from the raw data, as well as from the averages of margin types in each sampling site. Sex and species do not seem to predict the differences seen in this trend between specimens. The male *H. melpomene* CuA2 cell exhibits a more distal point of transition between flat/rounded margins and tines, with a broader period of mixture between the two. For the *H. erato* male, there is also a fairly wide area where flat/rounded margins and tines intermingle, but many scales with tines are found much more proximally, at around site 4 (as compared to site 8) (Figure 16).

The female *H. erato* had perhaps the weakest shift in margin type in the CuA2 cell, with large amounts of flat/round present until the very edge of the wing. The *H. melpomene* female, on the other hand, showed a strong trend with a neat, well-defined transition near the middle of the cell (Figure 17).

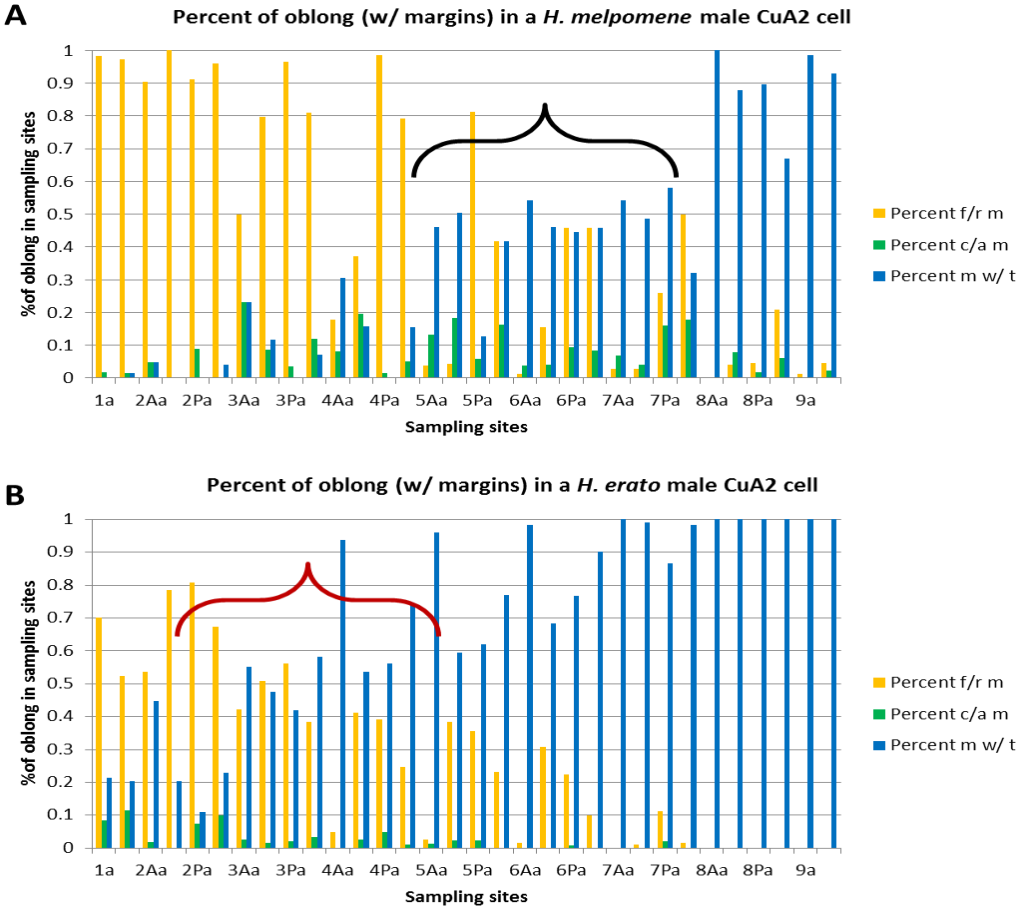

Figure 16.) Comparing the distribution of oblong scale margin types in the CuA2 cells of a male *H. melpomene* (**A**) and *H. erato* (**B**)**.** The area of transition between flat/rounded margins occurs more distally in **A**, indicated by the black bracket. In **B**, this shift occurs much closer to the wing joint, indicated by the red bracket. Total scale count=2523 and 2959; average scales per sampling site=79 and 92 for *H. melpomene* and *H. erato* respectively.

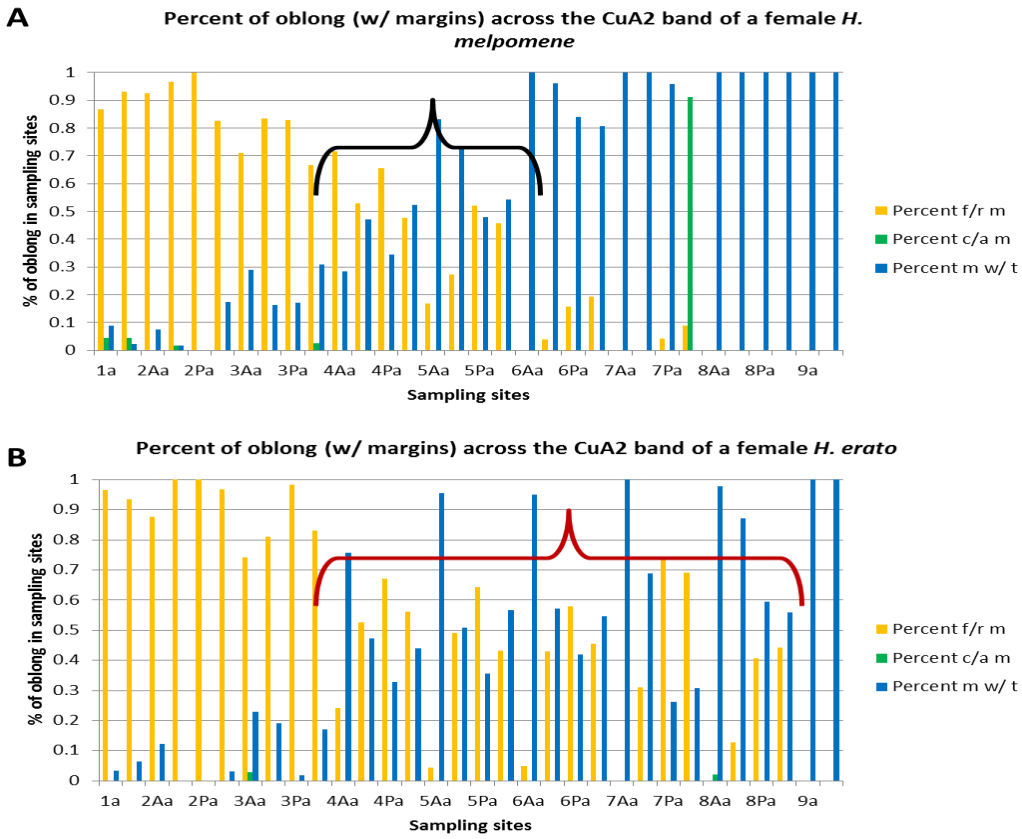

Figure 17.) Comparison of the distribution of oblong scale margin types in the CuA2 cell of a female *H. melpomene* (**A**) and *H. erato* (**B**). The area of transition between flat/rounded margins and tines occurs at a similar distance across the wing (indicated by brackets), but the prevalence of flat/rounded continues much more distally in the *H. erato* (shown by the red bracket). Total scale counts= 1911 and 1336; average scales per sampling site=60 and 41 for *H. melpomene* and *H. erato* respectively.

The Sc+R1 cells of the males displayed this pattern quite clearly, and also showed the trend seen in the CuA2 cell where the *H. melpomene* had a distal transition point and the *H. erato* a proximal one. The females of both species, however, lack clear transition points. In *H. melpomene*, flat/rounded margins make up most of the oblong scales until the very last sampling sites. Margins with tines, while present throughout the cell, do not become common until the edge of the wing. The *H. erato* female seems to have no transition point whatsoever. The

percentage of flat/rounded margins is very high at the most proximal and distal sites, then falls to below 50% for the majority of the cell. The scales with tines mirror this, with very low levels at the ends of the cell, but a percentage around 60% for the middle portion.

The margins of acute scales:

Acute scales comprise the majority of scale types along the margins of the wing, and seem to only be found in the marginal cells, 2A and C. Almost all acute scales have sharply edged, acute margins, but they can be more rounded as well (Figure 8). Only one specimen, the *H. erato* male, had an appreciable, but still small, number of acute scales with tines. While there does seem to be a proximo-distal shift in margin types, it is less easily observed than in oblong scales, as there are fewer acute scales. The average proportion of acute scale margin types in each sampling site shows this trend more clearly than the primary data. For example, the Au cell of the male *H. melpomene* specimen shows average percentages of convex/acute margins of 54% and 12%, and averages of rounded margins of 32% and 51% in the proximal and distal regions, respectively (Figure 18). This trend is more pronounced in the male specimens. It does exist in the females, but the average percent of acute scales in the distal regions of these specimens is very low, which makes patterns more difficult to see (Figure 19). No shift in margin type seems to occur along an anterior/posterior access—convex/acute are more common, but both these and flat/rounded margins decrease in accordance with the overall percent of acute scales (Figure 20).

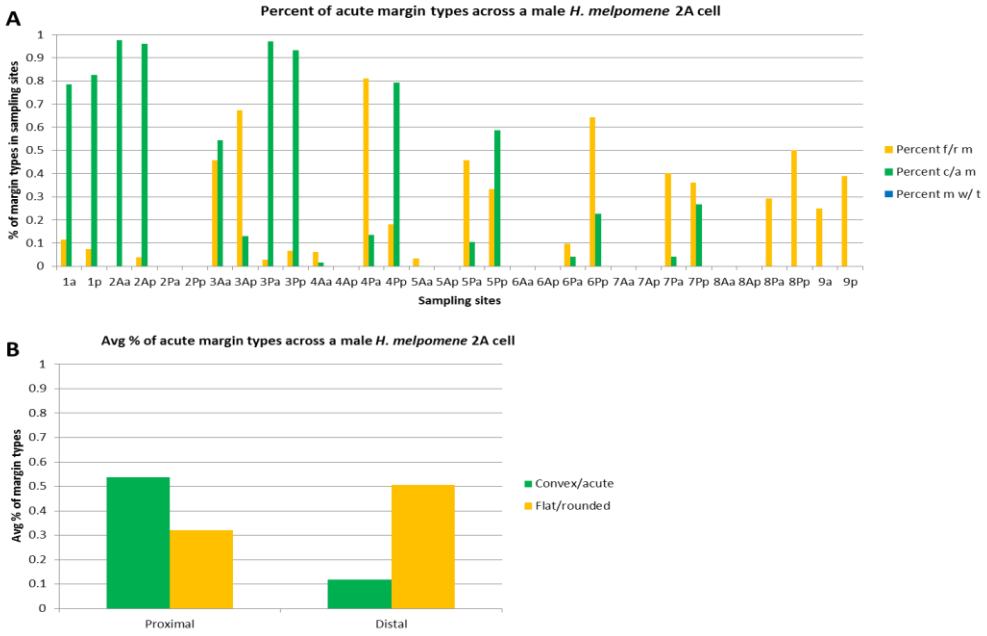

Figure 18.) Margin types of acute scales in a male *H. melpomene* forewing 2A cell. **A**--the percent of margin types in each sampling section along a proximo-distal axis. **B**—the average percent of acute margin types in the proximal and distal halves of the cell. Both **A** and **B** display a trend of a high percentage of convex/acute margins near the wing joint, shifting to more flat/rounded near the distal edge of the wing. Total scale count=1797; average scales per sampling site=60.

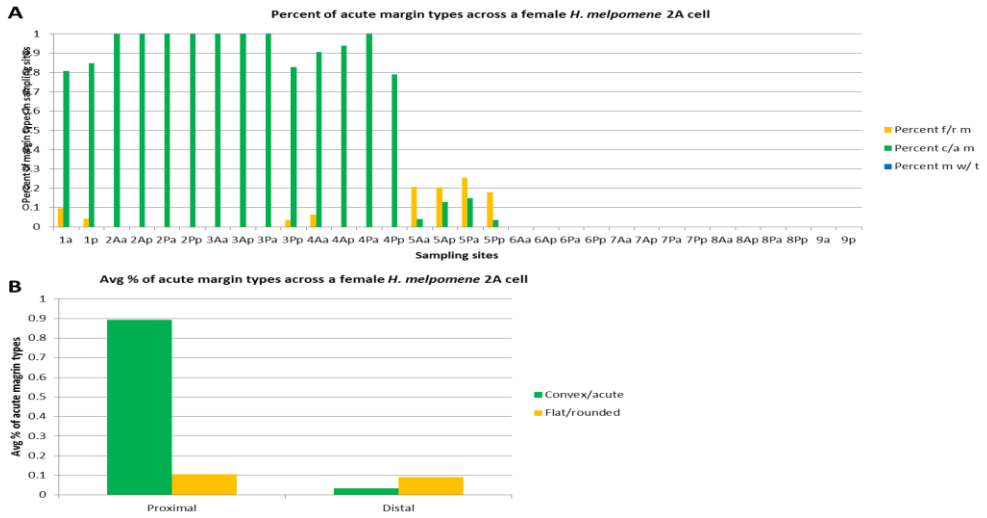

Figure 19.) Margin types of acute scales in a female *H. melpomene* forewing 2A cell. **A**--the percent of margin types in each sampling section along a proximo-distal axis. **B**—the average percent of acute margin types in the proximal and distal halves of the cell. Both **A** and **B** display a trend of a high percentage of convex/acute margins proximally, and low flat/rounded margins overall . This is most likely due to the short length of the female overlap band. Total scale count=1797; average scales per sampling site=60.

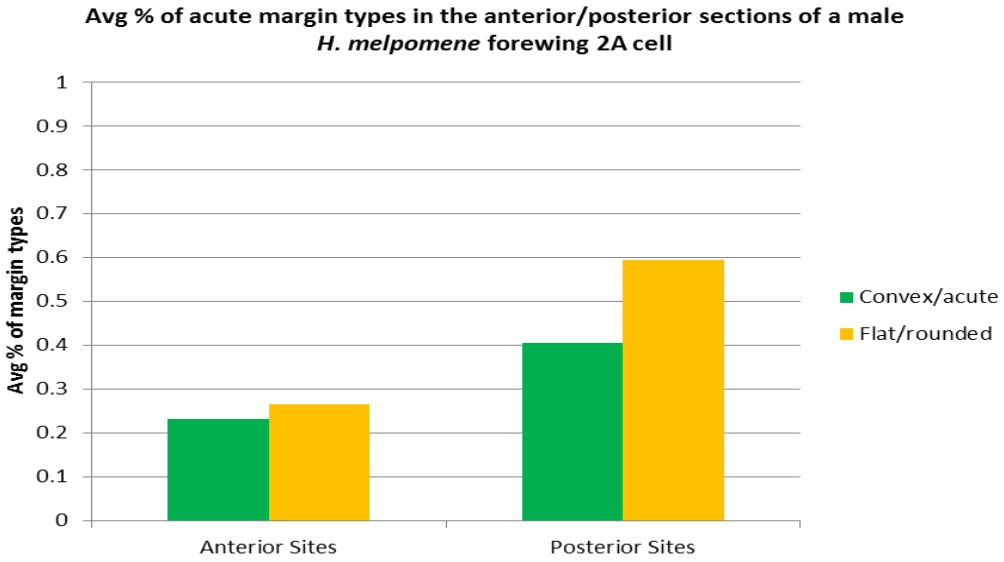

Figure 20.) Average percent of acute margin types in a male *H. melpomene* 2A cell. No obvious shift in distribution seems to occur. Both convex/acute and flat/rounded margins increase in the posterior half, though flat/rounded increase more to a greater extent. Total scale count=1797; average scales per sampling site=60.

Placement/structure of Androconia:

In concordance with published descriptions of *Heliconius*, I found that, within the regions I sampled, androconial scales occurred only on the hind wings of male butterflies of both species. The morphology of androconia is often species specific, which I found to be true in my specimens (Figure 21). In *H. melpomene*, the androconia were usually very rectangular in shape, with straight lateral edges, and little tapering. The hair-like extensions on the margin often seemed somewhat clumped, especially near where they diverge from the body of the scale, so that it may be plausible to postulate that they evolved from modified tines. The base of the scale showed a rounded, concave morphology, which was quite often asymmetric. The pedicel, the structure of the scale that attaches to the socket cell and attaches the scale to the wing, was unusual in that it looked quite flexible, and

was often curved and twisted. The pedicles of other scales appear to be quite stiff. The androconia of *H. erato* were often shorter and squatter than those of *H. melpomene*, and while they also had very straight lateral edges, showed a tendency to taper towards one end or the other. The hair-like extensions flared more, and were usually less clumped. The bases of these androconia were very deep, with lobes that were often tipped with small points that almost resemble the extensions on the distal end. Unlike *H. melpomene*, the pedicels always appeared stiff and inflexible (Figure 21). All scales have pedicels, but they often break off during sampling, so that not all of my images had this structure intact.

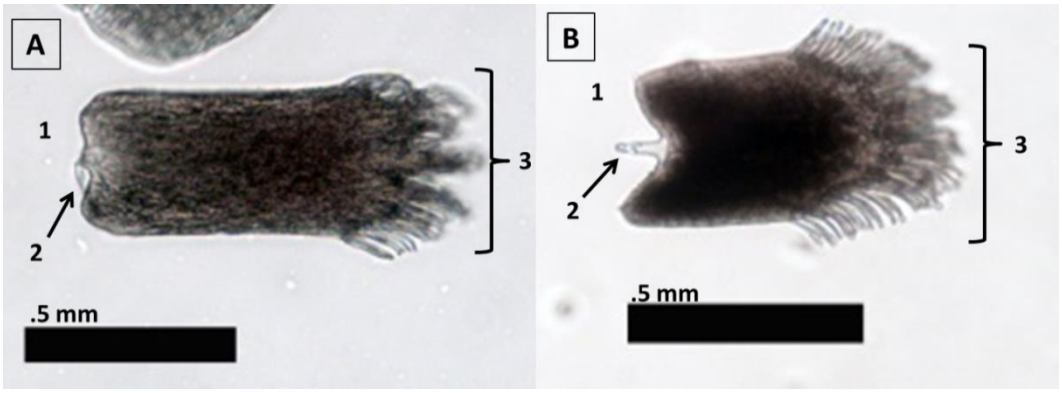

Figure 21.) Comparing androconia between species. **A** is from a male *H. melpomene*, and **B** is from a male *H. erato*. **1**—indicates the base, or proximal end of the scale. **2—**shows the pedicle, which attaches the scale to the wing membrane. **3—**indicates the hairy extensions that most likely help distribute pheromones. Scale bar is .5 mm.

One specimen each of two species prohibits any generalizations for *H. melpomene* and *H. erato*, but my results indicate that pupal-mater (*H. erato*) has fewer overall androconia than the adult-mater (*H. melpomene*). In *H. melpomene*, the highest incidence of androconia proved to be 63.4% in subsection 3Pp of the Sc+R1 cell, with a mean percentage of overall androconia in the hind wing being

19.2%. The male *H. erato* displayed a comparable highest percentage of androconia (63.6% in C cell, in the most posterior subsection of the 5th site), but had an lower overall mean percentage of androconia (13.8% for both C cell and Sc+R1). In both *H. melpomene* and *H. erato*, the concentration of androconia were heaviest close to the wing veins (Figure 23).

In both species, the highest mean percentage of androconia was concentrated in the Sc+R1 cell. This trend continues, in *H. melpomene*, at least, when the cells are broken down into their anterior and posterior sections (Figure 22). In *H. erato*, there is a higher average percentage of androconia in the posterior sections of the C cell, but the overall average is higher in Sc+R1. I conclude that this somewhat paradoxical occurrence is due to a higher average of androconia in both the anterior and posterior sections of Sc+R1, which, when combined, is higher than that in the C cell, even though there is a higher average of androconia in the posterior sections of the C cell.

When the cells of both species that contain androconia are broken down into the four subsections that I sampled (Aa, Ap, Pa and Pp), the average percentage of androconia are generally highest in in the most posterior subsections (Figure 24). As these cells are in the hind wing, these subsections abut the Sc+R1 vein in the C cell and Rs vein in the Sc+R1 cell. This indicates that the androconia are most highly concentrated along the wing veins. Both *H. melpomene* and *H. erato* display a trend across the C cells where the average

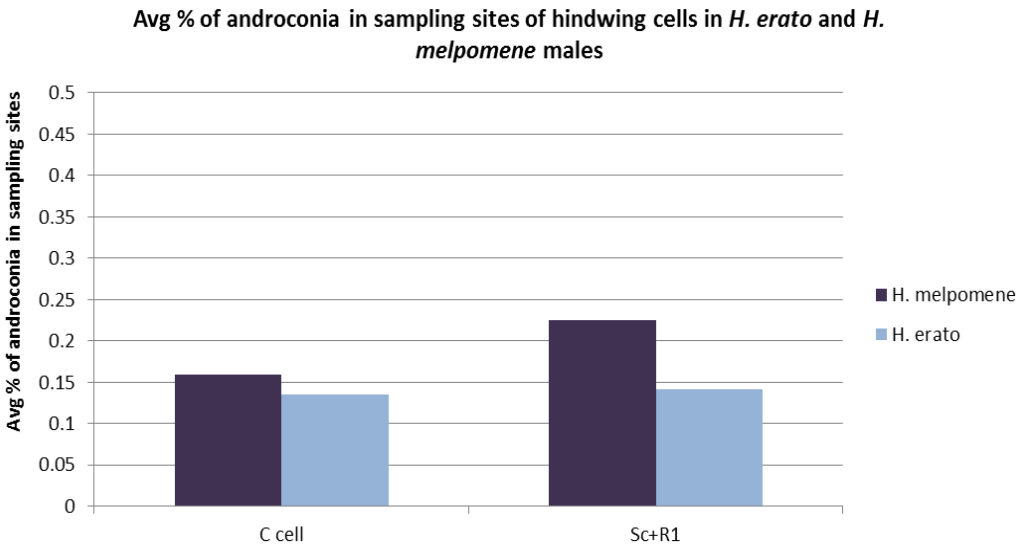

Figure 22.) Average percentages of androconia in the C and Sc+R1 cells for both *H. melpomene* and *H. erato* males. The average percentage of scale types made up by androconia is greater in the Sc+R1 cell of both species, though the difference is more noticeable in *melpomene*. Averages were calculated for scale counts within single specimens (C cell=2497; Sc+R1=2735 and C cell=3307; Sc+R1=3393 for *H. melpomene* and *H. erato* respectively).

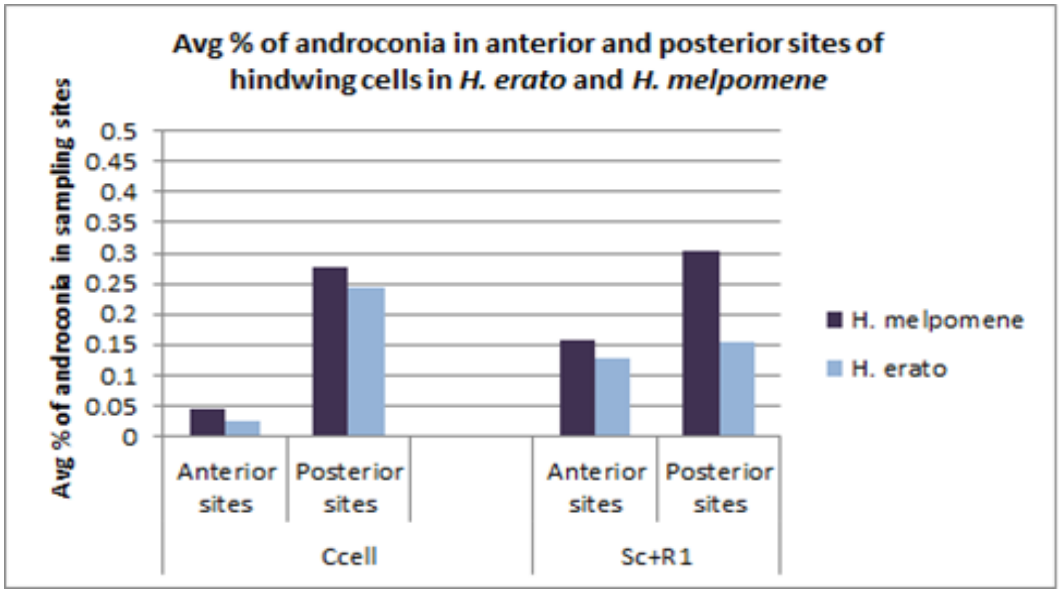

Figure 23.) Average percentages of androconia in the pooled anterior and posterior sampling site sections of the hind wing C and Sc+R1 cells of *H. melpomene* and *H. erato* males. Percentages are highest in the posterior sites of both cells, and overall higher in the Sc+R1 cell for both species. *H. erato* seems to have lower percentages of androconia than *H. melpomene*, but these averages are only from one specimen from each species (C cell=2497; Sc+R1=2735 and C cell=3307; Sc+R1=3393 for *H. melpomene* and *H. erato* respectively).

percentage of androconia is lowest in the anterior subsections, and increases posteriorly. The interior cell, Sc+R1, displays a more complicated trend. The highest average percentage of androconia still occurs in the most posterior subsection, but a striking number of androconia can also be found in the most anterior subsection. It is in the middle subsections, Ap and Pa, that the percentage of androconia is usually at its lowest. This again provides support for the hypothesis that androconia are most heavily concentrated along the wing veins (both Sc+R1 and Rs).

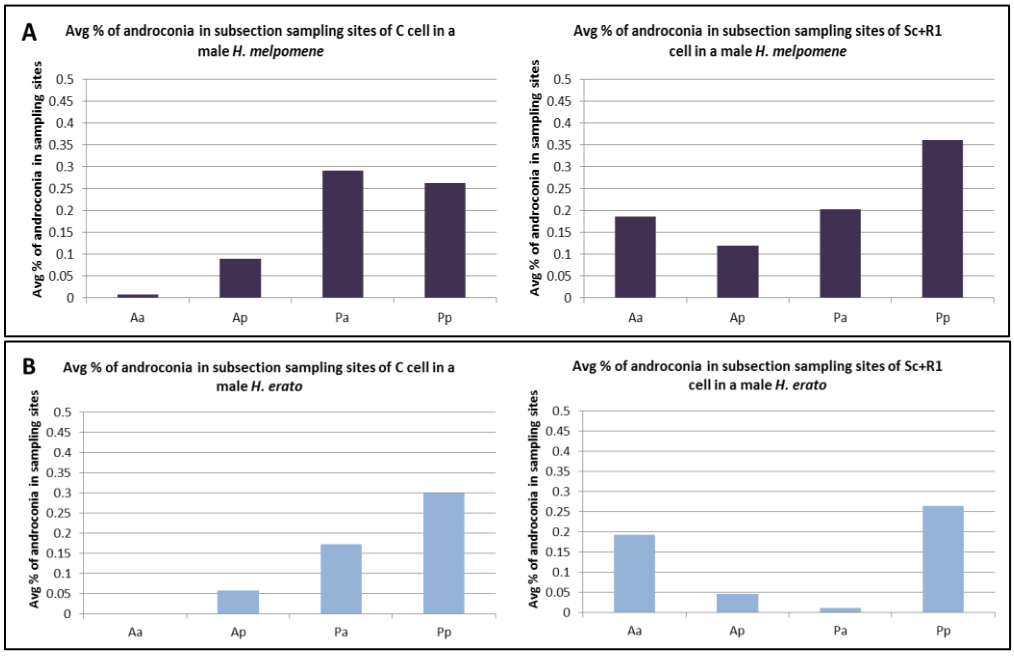

Figure 24.) A: Average percentage of androconia counted in each subsection of both hind wing cells of a male *H. melpomene* along an anterior-posterior axis. In the marginal C cell, the concentration of androconia increases posteriorly along the cell towards the Sc+R1 vein. In the Sc+R1 cell, which is interior, and therefore bordered both anteriorly and posteriorly by wing veins, the highest percentages are found in the Aa subsections close to the Sc+R1 vein, and in the Pa and Pp subsections, which are in close proximity to the R1 vein. **B:** Average percentages of androconia in a male *H. erato*, which shows trends similar to the *melpomene*. It has an even more pronounced decrease in androconia in the midsection of the Sc+R1 cell, and overall fewer androconia. Averages calculated from single specimens, with a scale count of C cell=2497; Sc+R1=2735 (*H. melpomene*), and C cell=3307; Sc+R1=3393 (*H. erato*).

Transitional or undeveloped androconium scale:

During the course of imaging scales, I ran into a few aberrant scales that did not fall into any of my categories. Most of them did not seem to have much to offer beyond my curiosity as to how or why they developed so oddly. One, however, proved to be quite interesting. On the hind wing of the male *H. erato* specimen, I discovered what appeared to be either a mixture between an androconium and an "asexual" scale, or an undeveloped androconium (Figure 25). I found this odd example in 6P (the 6th sampling site, posterior section) of the costal, or C cell. Unfortunately, the scale proved to be difficult to focus, but I was able to capture an image that shows most of the interesting features clearly. Androconia in *H. erato* have a distinctive shape in addition to the hair-like extensions on the margin described earlier (Figure 21). The basic shape of the transitional/undeveloped scale resembles a normal androconium, but is less well defined or well formed. The overall contour is rectangular, though asymmetrical, with the distal end wider than the proximal. The base lacks the depth or definition that most *H. erato* androconia display, and is very asymmetrical. The most intriguing feature, however, are the hair-like extensions. Not only are they much shorter than usual, but they do not flare out from the margin. In fact, they seem almost "stuck" together, as though they have not been fully divided. The pedicle, however, seems fairly normal. This gives the scale the appearance of a scale cell that died before forming into a fully-fledged androconium (Figure 25).

Figure 25.) A scale from the C cell of a male *H. erato* that seems to be either part way between an asexual scale and an androconia, or an undeveloped androconium. The overall shape resembles that of an androconia, if to a lesser degree than a fully formed one. The hair-like extensions (**3**) on the margin are much shorter than normal, and not as separated. The base (**1**) is not as deep as is typical for *H. erato* androconia, and is highly asymmetrical. The pedicle (**2**) seems fairly normal. Scale bar is .5 mm.

DISCUSSION:

The scale counts from my specimens point to patterns and trends in the distribution of scale shape across the overlap band. In both species, the shifts in distribution occur along both a proximo-distal and, less obviously, an anterior-posterior axis. The frequencies of different scale types fluctuate in similar ways in regard to both sex and position in the overlap band, though there is a fair amount of variation between specimens. Other than the general morphology of the androconia, no diagnostic or consistent differences could be seen between the two species *H. melpomene* and *H. erato*.

My research resulted in many data points, but they come from one male and one female each from two species. Because of this, my analysis mainly lies outside the realm of statistics. Future work involving the overlap band could go in two main directions: either repeating the methods used in this study on a statistically significant number of samples with appropriate controls, or an exploration into what the trends and patterns I observed can reveal about the development of scales across the wing. Both routes could provide valuable and important insights into the biology of *Heliconius*. Investigating the scale shapes of the overlap band in more subspecies from more locations could not only corroborate what I found, it could also expand and solidify the trends I observed, and could potentially reveal more aspects of the story that were not revealed with my few samples. On the other hand, studying the development and organization

of the overlap band could lead to a deeper understanding of how scales and color patterns are formed in the pupae to give the adult wing its proper appearance and function.

The formation of wing scales involves complex interactions between cytoskeleton, pigment accumulation within a cell, chitin secretions outside the cell, interactions with the socket cell, and the genetic and developmental pathways for the morphology and spatial distribution of scales. This thesis could function as the basis for not only a broader examination of the overlap band between adult and pupal-maters, but also for a study of how the morphology and placement of scales interact in development. In one of the central studies on *Heliconius* wing scales, Gilbert *et al*. (1988) determined that the scales fell into three main categories, depending on their pigmentation and ultrastructural, cuticular architecture on the external and interior surfaces of the scale. Type I scales are white which lack pigment or yellow, that are colored by a chemical called 3-hydroxykynurenine. Black scales make up type II, and are pigmented by melanin. Type III scales consist of red and brown scales colored by the chemical xanthommatin (Gilbert *et al*., 1988). Later research showed the strong correlation between scale ultrastructure and pigment by demonstrating that scales damaged or induced to change color during development switched their structure and type (Janssen, Monteiro and Brakefield, 2001). They showed that altered scales changed their architecture to that of the experimentally induced color (Janssen, Monteiro and Brakefield, 2001).

A recent study on the fine structure of the scales of the overlap band potentially complicates this picture. Despite the superficially apparent uniformity of color in this region, researchers have found that the ultrastructure of all three of Gilbert's scale types are represented in the overlap band, at least in males (Aymone, Valente and de Araújo, 2013). In *Heliconius*, the scales of the same type, colored by the same pigment, seem to mature at the same time during development. Red and yellow scales mature first, though pigment is synthesized the red first and the yellow after maturation. The black scales mature later than either of the other two types (Aymone, Valente and de Araújo, 2013). In this study they note that the scales of the overlap band mature at the same time as the black scales on the rest of the wing, which suggests that the color of the overlap band is caused by the same pigment: melanin (Aymone, Valente and de Araújo, 2013). The study hypothesized that pigment could play a bigger role in maturation than ultrastructure, since the overlap band scales consist of all three scale types and still mature at the same time as the type II, or black scales, instead of the red/brown or yellow/white types (Aymone, Valente and de Araújo, 2013). They speculate that the disjunction between scale pigment and ultrastructure could indicate that something intriguing is occurring in the scales of the overlap band during metamorphosis (Aymone, Valente and de Araújo, 2013).

By adding the overall shape of the scales into an examination of the developmental connections between pigment and ultrastructure, we might gain a better understanding of how the wing pattern is determined. Previous studies

(Kusaba and Otaki, 2008) and my own data establish that spatial position on the wing correlates with scale shape, and sometimes size. Location appears to be important in the overlap band, where the prevalent scale morphology changes depending on location within the region. Studying how spatial positioning also ties into pigmentation, surface structure and shape could illuminate how so many disparate factors connect and communicate in order to form the correct type and color of wing scale for a specific site on the wing. This could be important, not only for pattern formation, but also for the aerodynamics of the wing. The shapes and layering of scales can affect how the air flows over the wing, which in turn affects the flight of the insect.

Throwing in yet another layer of complication, studies exploring the effects of the gene *optix* indicate that it has strong effects both on the placement of red-pigmented scales in *Heliconius*, and the formation of scales with an acute morphology in this genus and in other nymphalid butterflies (Reed *et al*., 2011). Acute scales, with their sharp, often hooked morphology, are thought to function as grappling hooks that hold the fore and hindwings together during flight. This could serve as an explanation of why they are mainly found along the proximal edges of the overlap band. By examining the transcription of this gene, the location of red color pattern elements and patches of acute scales can be accurately predicted (Reed *et al*., 2011). The determination of red or acute scales could possibly be determined by a simple switch, inferred from the presence of *Heliconius* mutants presenting acute, non-red scales in the midst of a red color

pattern (Reed *et al*., 2011). The overlap band, with its abundance of acute scales, could illumine how one gene can determine either pigment or morphology, and how such genes interact with spatial information in order to make the correct decision.

The presence of androconia in the overlap band presents more questions concerning scale development. From my observations, androconia often occupy the space normally filled by ground scales, underneath the larger cover scales. Perhaps androconia in *Heliconius* evolved from, or replace, the ground scales. The question of how different scales, such as cover or ground scales, are organized and develop in the same area to create the dual layering seen on most of the wing, is still not answered. On the reverse side of such a question, how do areas develop like the inner and costal margins, where only a single layer of acute scales occur? The presence of androconia, the uncoupling between pigment and ultrastructure, and the strong trends in scale shapes all lean toward the possibility that the overlap band is doing something different from the rest of the wing during development.

Several models for how the scales across the wing are organized during metamorphosis have been presented in the literature on Lepidoptera. Despite the amount of research that has been conducted on butterfly wing patterns, contention remains surrounding the presence of organizational centers and how they function, be it through single or complex morphogen gradients, or cellular induction (Nijhout, 1991; Otaki, 2008; Otaki, 2011). Nijhout, in his examinations

of wing color patterns across a multitude of moth and butterfly families, postulated that the wing veins organize the wing during development (1991). Since the veins provide a route for nutrients via hemolymph, and are congruent with the path of pupal nerves, there are multiple, possibly complementary ways that they could be affecting scale provisioning and patterning. Each wing cell, or region between the veins, seems to have independent "control" of its color pattern. Several studies have hypothesized that the certain areas, such as wing veins and eyespots, serve as center of organization during development that could determine color, ultrastructure, size and morphology. (Nijhout, 1991; Janssen, Monteiro and Brakefield, 2001). This has been most often examined for color, but holds potential explanations for the organization of scale shape as well. Some contrary evidence argues that veins do not fully control wing patterns, at least in *Heliconius*. The discovery of a *Heliconius cydno* mutant with severely reduced veins, but close to wild-type patterning, presents the possibility that color patterns in this genus develop independently of the wing veins (Reed and Gilbert, 2004). Perhaps the development of the wing pattern is so well canalized, that it can form normally even in the absence of wing veins. Both generally in lepidopterans, and in *Heliconius* specifically, the mechanisms of organization for color patterns remain an aspect of development that is not fully understood.

Several trends shown in my analysis seem to be consistent with Nijhout's idea of wing veins serving as organizational centers. Often, the two sampling subsections located along the interior of the cells, Ap and Pa, had much more in

common than the subsections closest to the wing veins. Surprisingly, the most anterior and most posterior subsections of the sampling sites often displayed quite similar compositions of scale types. This does not hold true for the marginal wing cells, where there is only one vein border. Strong anterior/posterior trends are also apparent in these cells, which would be consistent with the wing veins serving as organizational centers. This could possibly be an artefact, however, where scale shape is more correlated to the aerodynamics of the wing, rather than the wing veins. The prevalence of androconia along the edges of the wing cells lends another piece of evidence that could support this theory. Androconia are concentrated most heavily along the veins of the overlap band, and gradually diminish in frequency towards the interior of the cells. Perhaps the release of a hormone from the veins during scale development could induce ground scales to become androconia in a graded fashion that tapers off as the organizational factor becomes less concentrated.

However, unless the organizational factors released by the wing veins also have a proximo-distal component, it cannot explain all of the variation of scale shape seen in the overlap band. While shifts in the distribution of scale shapes were seen along an anterior/posterior axis, especially with androconia in the males and acute scales in the marginal cells of both sexes, the strongest consistent trends appear to be from proximal to distal. It seems reasonable to assume, based on this, that another method of organization exists, set up along a proximo-distal axis. It has been postulated that such an axis could be set up in the larval wing imaginal

disks, and could play a large role in pattern development, though the idea remains mostly unexplored (Joron *et al*., 2006a). From my data, it seems that explanations combining these two proposed mechanisms for wing organization could contribute to the development of the scales in the overlap band.

My results point to several strong trends in the distribution of wing scales that exist across the overlap band. The differences between these trends seemed to be minimal between the two species, which, due to how distantly related *H. melpomene* and *H. erato* are within the genus *Heliconius*, raises some interesting questions. The differences between males and females, however, proved to be more difficult and complex to unwind. While an obvious large-scale dimorphism exists in the overlap band, which is what drew my interest in the first place, the differences on a finer scale are, for the most part, more subtle. The one obvious sexual dimorphism in regards to scales is the presence of androconia on the hind wings of the males. If one removes these sex-specific scales from the overall analysis, only subtle, inconsistent dimorphism remains on the level of scale shape. The Sc+R1 and the CuA2 cells of the hindwing and forewing, respectively, display oblong scales almost exclusively in both sexes once the influence of androconia has been removed. However, males and females do seem to differ in their distribution of oblong margin types in the Sc+R1 cell. Males of both species show a strong shift from flat/rounded margins proximally, to margins with tines distally. In females, there is no proximo-distal shift in margin type. Roughly half of the oblong scales have flat/rounded margins, and roughly half have margins

with tines for the majority of the cell. The other interior cell, CuA2, shows no sexual dimorphism between males and females.

Some sexual difference does linger in the marginal cells, where the females show a much stronger shift from acute cells proximally to oblong scales distally. This, however, probably has less to do with sexual dimorphism on the cellular level of the scale, and more to do with the fact that the female has a short and narrow overlap band. In both males and females, scales that more closely resemble those found in the main pattern elements on the rest of the wing begin to encroach on the overlap band near the distal edge of the wing, and where the band merges with the rest of the color pattern. My scale count data only took into account the general scale shape, not its "affinity," by way of color and morphology, for either the overlap band or the main pattern elements. Given that most main pattern element scales tend to be oblong, this could have skewed my data to indicate a strong distribution trend in the marginal cells of the females. The percent of acute scales falls to zero a little more than half way across the cell (around the most proximal subsection of the 5^{th} sampling site), leaving the rest of the wing to be dominated by oblong. More analysis, focusing on the specific differences between overlap band and main pattern element scales would be necessary to determine exactly where the oblong scales of the overlap band give way to those found on the rest of the wing.

Even though this is a very preliminary study, it raises intriguing questions about a well-studied organism. According to the *Zoological Records*, 700 papers

have discussed *Heliconius* from 1864 to the present, with 209 published within the last 10 years. This impressive literature includes only 5 studies in the last decade that mention sexual dimorphism, and none of those involve the overlap band. Some discussed differences in taste receptors—important in females for choosing host plants—while others examined possible sexual dimorphism in the bands of the main, aposematic color patterns. The one paper that has looked into the scales of the overlap band focused mainly on the surface architecture and pigment of the scales, rather than the overall scale shape, and did not take sex into account (Aymone, Valente and de Araújo, 2013).

A dearth of research also exists on the subject of courtship, pheromones, androconia, and how they interact in *Heliconius*. The mechanics of *Heliconius* courtship and copulation, except in a few species, remains largely undescribed in the literature, though the presence of androconia in the overlap band indicates that it plays a role. Most of the research done on pheromones in the genus revolves around antiaphrodisiac compounds (Estrada *et al.*, 2011; Klein and de Araújo, 2010). Since females often mate repeatedly throughout their long lives, at least in the adult-mating clade, males appear to have several tactics to gain precedence for their sperm and prevent the female from re-mating for as long as possible (Cardoso, Roper and Gilbert, 2009). These approaches include antiaphrodisiac pheromones and large spermatophores. Antiaphrodisiacs, when released passively by females, indicate to males that she has recently mated and is unreceptive to courtship, protecting her from undue hassling from unwanted mates (Estrada *et*

al., 2011). Males, however, have taken advantage of these compounds, and transfer them to females during copulation, thereby warding off future competing males (Estrada *et al*., 2011). By transferring a large spermatophore upon mating, the male increases the females' refractory time between matings, therefore increasing the chance that his sperm will be used in fertilization (Cardoso, Roper and Gilbert, 2009).

Research conducted on *Heliconius* androconia and their associated pheromones seems not to exist. In fact, studies on androconia in general are few and far between. The general consensus is that they are specific to males and distribute short range pheromones during courtship. It is assumed that both sexes use this in species recognition, which might be particularly useful where many congeners both mimic each other and are sympatric. A few different strategies seem to exist among different families and genera of Lepidoptera. The most common type of androconia includes those found in *Heliconius*. These scales are usually long and thin, often with unusual morphologies, (in this aspect, *H. melpomene* and *H. erato* seem to be something of an aberration with short, squat androconia), and are tipped with hair-like extensions (Kristensen and Simonsen, 2003). The method of pheromone dispersal appears to be evaporation, with the scented secretion being drawn up from the base of the scale through capillary action to disseminate at the distal end (Kristensen and Simonsen, 2003). The other main strategy, which seems less common, involves long, tubular scales, often called "hair pencils," that break upon contact and release the pheromones stored

within (Kristensen and Simonsen, 2003). The timing and production of chemical substance itself is uncertain, but the prevalent hypotheses surmise that it is synthesized either at the base of the androconia by surrounding epithelial cells, or elsewhere in the body and then transported to the scale (Kristensen and Simonsen, 2003). Further than this, however, the subject seems to be little understood. How they develop, what the genetic and developmental pathways are that control their placement on the wing, and how they interact with other scale types, remain shrouded in uncertainty.

How are scales triggered during development to become an androconium rather than a normal wing scale? Does this process happen early in development of scales on the wing, or late? Does it occur at the same time, and is controlled by a gene or hormone or signaling molecule found only in males? My own observations suggest that these androconia were often overlaid by cover scales. This could indicate that, at least in the two species I studied, they were exapted from ground scales. Exactly how the pheromone distributed by the androconia becomes associated with the scale is also somewhat uncertain. One possible method postulated in the literature involves epithelial cells adjacent to the socket cell at the base of the scale producing the pheromone, which is then transported to the end of the androconium by capillary action (Kristensen and Simonsen, 2003). During the course of my study, I noticed that there seemed to be a raised structure along the wing veins that was a slightly different color from the rest of the cuticle. I did not have time to look into this further, and did not note whether I saw this

only in males, or in both sexes. Since androconia seem to be more heavily concentrated along the wing vein, this barely characterized structure could be involved in their formation or function.

The odd scale I found on my male *H. erato* specimen provides a place to begin asking questions about how a panoply of scale types exist in a small area, and yet are parts of an ordered pattern. It seems reasonable to expect that androconia evolved from normal, "asexual" wing scales. They are highly specialized structures found only in one sex, which makes it improbable that they would be the ancestral trait. The most parsimonious explanation would predict that androconia were derived from asexual wing scales, and adapted for the function of distributing pheromones. This hypothesis could be supported by the transitional scale I discovered. The hair-like extensions appear fused together along the margin, which resembles, in general shape, the distal end of an asexual scale much more closely than that of an androconium. The rest of the scale, however, much more closely resembles a normal androconium. This could indicate that androconia begin developing much like a normal scale, and accumulate the characteristics of a sexual scale as the cell matures. To determine if this is actually the case, a study could be conducted to examine the wings of developing males in areas where androconia are known to be present in adults. By dissecting out and examining the wings at different stages of pupal development, one could gain a sense of how the androconia form from the living scale cells.

My examination of the overlap band resulted in a few partial and tentative answers and even more questions. The strong trends in the distributions of scale shapes in the overlap band provide an opportunity to explore the patterns of developmental organization across the wing. The apparent disconnect between the pigment and ultrastructure of the scales, which seems to be closely linked across the rest of the wing, indicates that the overlap band is breaking some of the rules that have been proposed for scale development in *Heliconius* (Aymone, Valente and de Araújo, 2013). Much as mutants provide the path to understanding the function of a wild-type gene, the overlap band could serve as a natural experiment revealing an alternative to the developmental pathways that connect the pigment and ultrastructure of scales in the rest of the wing in *Heliconius*. The scales of the overlap band are not warning off potential predators or mimicking other species, but rather serving as communication between the sexes. The presence of androconia and the role of the overlap band in courtship also offer rich areas of study. This could be approached in a developmental and cellular approach aimed at the formation of androconia, from a physiological and chemical angle examining the male-specific pheromones, or from the perspective of evolution and ecology by attempting to understand the role of the overlap band in courtship and its escape from the constraints of mimicry.

Beyond looking at the overlap band directly, several intriguing avenues of study could be pursued by following the questions raised by this research and possibly using some of the methodology employed by it. Fine-grain scale

sampling across and between wing cells could be applied in other areas of the wing for many purposes, such as trying to understand developmental organization and examining how scales change at the edges of different color pattern elements. *Heliconius* presents a plethora of questions to be asked along these lines. From Nijhout and Wray's work on the formation of *Heliconius* wing patterns, it is understood that the black areas of the wing are the actual elements of the nymphalid ground plan that spread out from "nuclei," or centers of the color pattern elements (1988). The red, white or yellow bands are the background colors, or the areas that the black pattern elements do not reach (Nijhout and Wray, 1988). It is possible that the black pattern elements can also switch to red, making it difficult to determine what is background and what is pattern (Nijhout and Wray, 1988). Reed *et al*.'s (2011) work on the *optix* gene and its connection to red coloration complicates this question even further. According to Gilbert *et al*.'s (1988) research, the ultrastructure and pigment of *Heliconius* scales are closely linked, but what about the overall morphology of the scales? Detailed sampling in areas that formed from either one or several merging pattern elements could show if scale shape differs due to origination of the color pattern. Comparisons between red scales might indicate alterations in shape depending on if the red pigmentation came from background that was not invaded by a pattern element, or from a switch between black and red elements. Perhaps there is no correlation, or a weak one, between scale shape and pattern element, and

morphology is determined solely by location on the wing. Either way, detailed studies of scale shapes could shed light on the subject.

The mechanisms that build the scales that I observed and analyzed remains, in large part, enigmatic. The genetic and developmental pathways that translate the products of the supergene into the actual shaped, pigmented scales in their appropriate locations on the wing are, as yet, largely unknown. Studies have indicated that the patterning supergenes are unlinked to the genes that determine scale pigment and their spatial organization, which hints at a complex interaction of genes and their products (Joron *et al*., 2006b). Whether or not sexual dimorphism occurs on the level of the scale, it can be seen in the overlap band that the shape can change drastically depending on the scale's placement on the wing. Delving into how, and potentially why, this phenomena occurs would be a useful object of study, and, from the characteristics I observed, the overlap band could serve as a beneficial model, especially since it remains largely free of the constraints of mimicry put on the rest of the wing.

The overlap band, a forgotten element of a well-researched genus, serves as a wellspring for new ideas about the organization and development of wing patterns in *Heliconius*. Riffarth and Kaye demonstrated the overlap band's usefulness in classification and the nature of co-mimicry in *Heliconius* through their study of its morphology (Riffarth, 1901; Kaye, 1907). My research into the sexual dimorphism of the overlap band and the shifting distributions of scale shapes within it, while far from conclusive, could provide a foundation for future

work. For me, at least, the enigmatic characteristics of these hidden pattern elements of the wings serve as an example of what we still do not understand in a genus that has been extensively studied for over a century.

The patterns on the wings of moths and butterflies are a microcosm for the study of evolution, ecology and behavior. *Heliconius* departs from the norm in almost every aspect of the Lepidoptera, including life history, nutrition, mating, and interactions with congeners. By understanding how they deviate, we can gain a greater understanding of the overall biology of moths and butterflies. Like a fractal pattern, the overlap band serves as a microcosm within and foil to *Heliconius*. It is where clade, sex, courtship, and scale shape can be studied, freed (at least to some extent) from the extrinsic demands of mimicry. These narrow bands are the foil to the foil, and, as such, could reveal new perspectives on these beautiful and entrancing insects.

APPENDIX:

Methods:

As a supplement to the scale count data, which relied on a rather qualitative interpretation of scale shapes, I here present a more statistical analysis on the morphology of the scales in the overlap band. To do this, I imaged between 5-20 scales from each subsection for use in a morphological analysis of scale shape. All the images were taken at 400x with brightfield microscopy with an Olympus BH-2 microscope, a PixelLink PL-B681CU camera and the program PixelLink OEM. In order to study the morphology of the scales, the edges of the scale had to be sharp, defined, and mostly unbroken. This means that I tried as much as possible to image scales that were perfectly focused (flat), undamaged, and not overlapped by other scales or dirt. If a scale seemed particularly interesting for some other reason, but did not meet the criteria for imaging, I would take a picture of it for reference. I took detailed observations for each scale I imaged, noting the overall shape, the margin type, the shape of the base and pedicel, whatever I could see of the ultrastructure, and any unusual features.

After I completed imaging my scale samples from each butterfly, I chose the five best pictures of individual scales from each anterior and posterior section of each sampling site. Originally I had planned to analyze two images from each subsection, but the difficulty of finding enough clear, undamaged and unobstructed images, plus the sheer number of pictures that would have had to be

analyzed lead to a reduction. I attempted to capture the range of variance in subsections, however, by making sure that the images for each section contained some scales from both the anterior and posterior subsections. I did not use any images of androconia from the male specimens, as I wanted to see if any trends could be seen in the "asexual" scales. Ideally, two analyses could have been conducted: one with androconia, and one without. Because of time restrictions, however, I only conducted an analysis lacking androconia. I analyzed a total of 360 scales per specimen, and 1440 for all four specimens. I processed these images using Adobe Photoshop for analysis by adjusting the contrast of the images in order to sharpen the outline of the scales, adding a scale bar shape and making sure all scales had the same orientation. After the processing was complete, I turned my image data over to April Dinwiddie for analysis.

Results:

A. Dinwiddie returned the first five axes of the Principal Component Analysis (PCA) conducted on the data set of the elliptical Fourier analysis. These cover approximately 90% of the variance seen in scale morphology. Figure 26 displays the characteristics described by the first five PCs, with the mean and +2/-2 standard deviations. The standard deviations give a sense of what feature of the scale is covered by each PC. The average of every axis is not distinguishable from each other, but when you move away from the mean, the PCs capture some aspects seen in the scales, such as width and angle.

A. Dinwiddie created a morphospace for three combinations of PCs: PC1/PC2, PC2/PC3 and PC3/PC4. In Figure 27, the background images are the theoretical morphologies predicted by the PCA, while the scales are shown as points on the morphospace with PC1/PC2 as the axes. This gives a graphic representation of the distribution of scale shapes found within the overlap band that are described by the first two PCs.

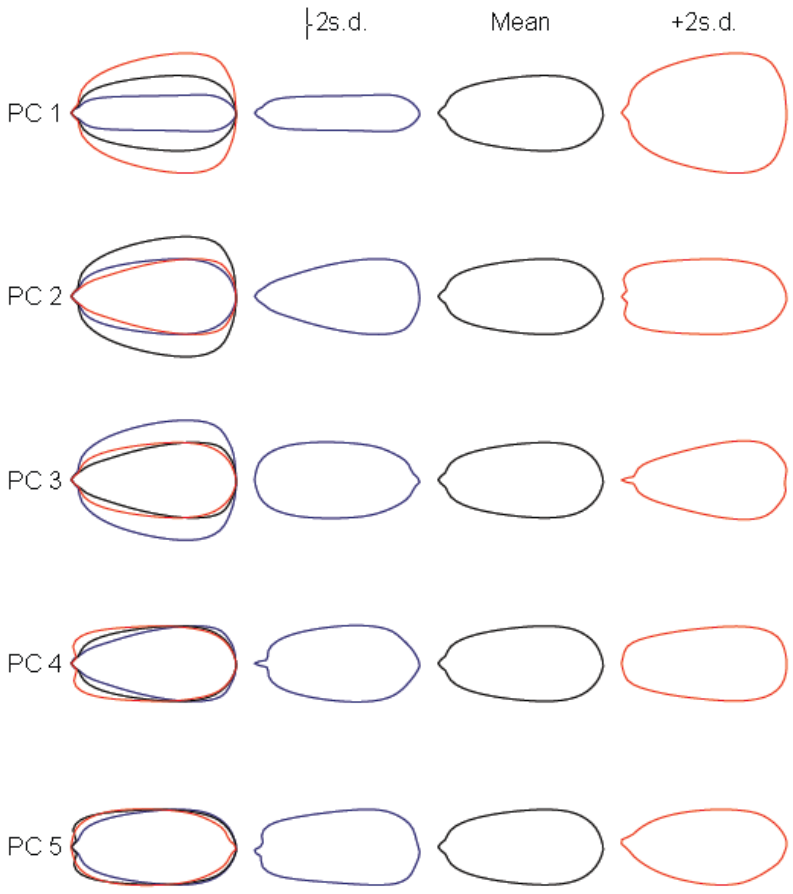

Figure 26.) The 5 principal components (PC) that describe the main variations seen in the scale shapes of the overlap band. The black outlines show the mean shape, while the blue outline is the -2 standard deviation, and the red is the +2 standard deviation.

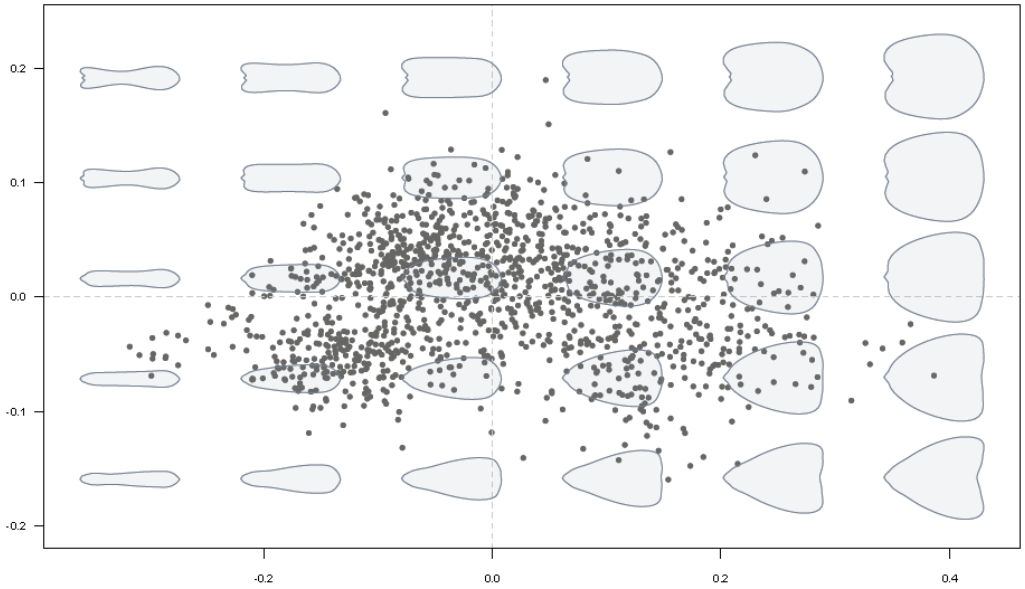

Figure 27.) The PC1/PC2 morphospace of the mean overlap band scales overlaying a theoretical morphospace predicted by PCA analysis. Each dot represents an imaged scale that was analyzed by elliptical Fourier analysis, and its location in the morphospace represents the characteristics described by PC1 and PC2.

In order to determine if scale shape differed by specimen, A. Dinwiddie created a PC1/PC2 morphospace that is color coded by the scale shapes found in each specimen (Figure 28). The areas in the center, where many scales are overlapped, show the common scale shapes possessed by all of the specimens. Where the layers of scales spread out along the edges give an idea of the morphologies that serve to separate the specimens. The two males seem the most different from each other, with the *H. melpomene* male being concentrated to the lower left of the morphospace and displaying small, narrow scales, and the *H. erato* male showing broad, rounded ovals or oblongs in the top right quadrant. The females of the two species appear somewhat similar—both are seen in the lower right portion of the morphospace, with broad oblong scales that could

almost be described as half ovals or rounded triangles. The *H. erato* female seems to trend toward the more oval scales of her male counterpart. It could be said the *H. melpomene* female does likewise, with a fair concentration of scales nearing the narrow, pointed morphologies that the male *H. melpomene* displays. These trends seem to be supported by Figure 29, where a convex hull has been placed around the range and average of the scale shapes for each specimen. The females of both species still seem to overlap in regard to their scale shape distribution, and are concentrated more in the lower right portion of the morphospace. The males of *H. melpomene* and *H. erato* seem to share more scale types in this representation, but they are still found higher in the morphospace than the females, and with their respective left and right tendencies. It is important to recall that the androconia, which would have tightly grouped the males together, were not included to see if patterns in the "asexual" scales would be revealed.

A. Dinwiddie conducted a similar analysis on the PC1/PC2 morphospace for the 4 different wing cells that make up the overlap band. In Figure 29, each color corresponds to one of the wing cells of the overlap band (2A and CuA2 in the forewing, C and Sc+R1 in the hindwing). Unlike the morphospace generated around the specimens, there seems to be less general overlap in the center, and more recognizable trends throughout the morphospace. The variation of the scale shapes seems to track whether the cells came from the forewing or the hindwing.

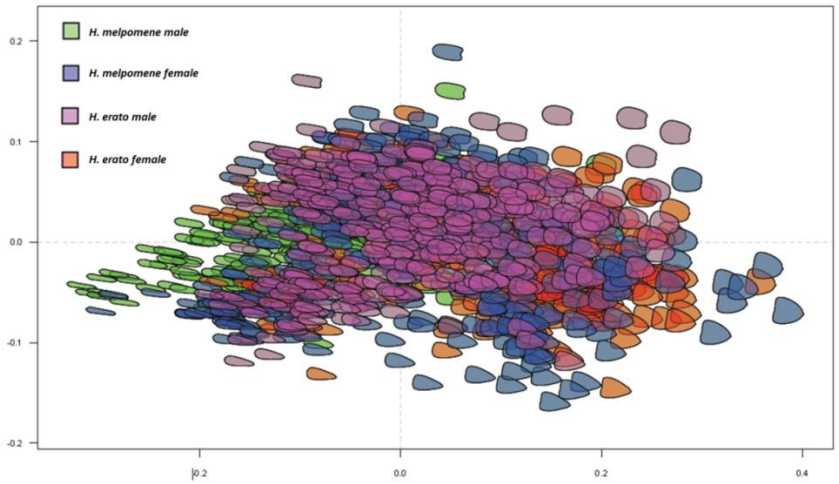

Figure 28.) PC1/PC2 morphospace color-coded by specimen. The overlapping in the center shows scale shapes common to all the butterflies, while the areas along the fringes show scale shapes unique to one or two specimens. The *H. melpomene* male seems to have a monopoly on thin, long scales, found on the far left of the morphospace (green). Both the *H. melpomene* and *H. erato* females seem to have broader oblong scales, that could almost be called triangular (blue and orange respectively). The *H. erato* male shows only a few scale shapes not seen in other specimens. These are broad, rounded ovals in the top right quadrant of the morphospace (purple).

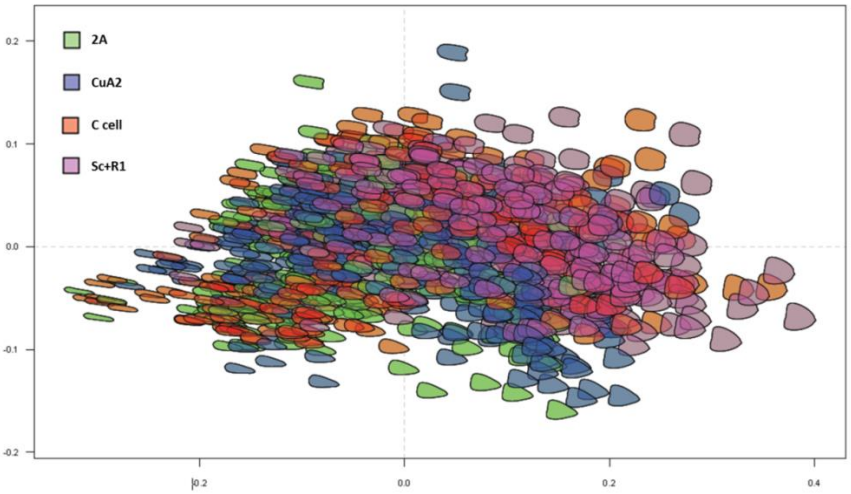

Figure 30.) The PC1/PC2 colored by the location of the scale shape on the wing. The variance seems to correspond, for the most part, with whether the cells are located on the forewing or the hindwing, rather than by similar cell types (interior versus marginal). The cells of the hindwing, Sc+R1 and C (Figure 4), are concentrated in the upper right quadrant, with mostly ovular scales ranging from broad to moderately narrow. The 2A and CuA2 cells of the forewing (Figure 3) make up the middle and lower right sections, with a broad range of scale shapes from rounded triangles to narrow oblongs. The variance in the far left of the morphosphere is dominated by the 2A and C cells, which are the marginal cells of the fore and hindwings, respectively. These cells occur along the long edges of the wing, and are where acute scale types are concentrated.

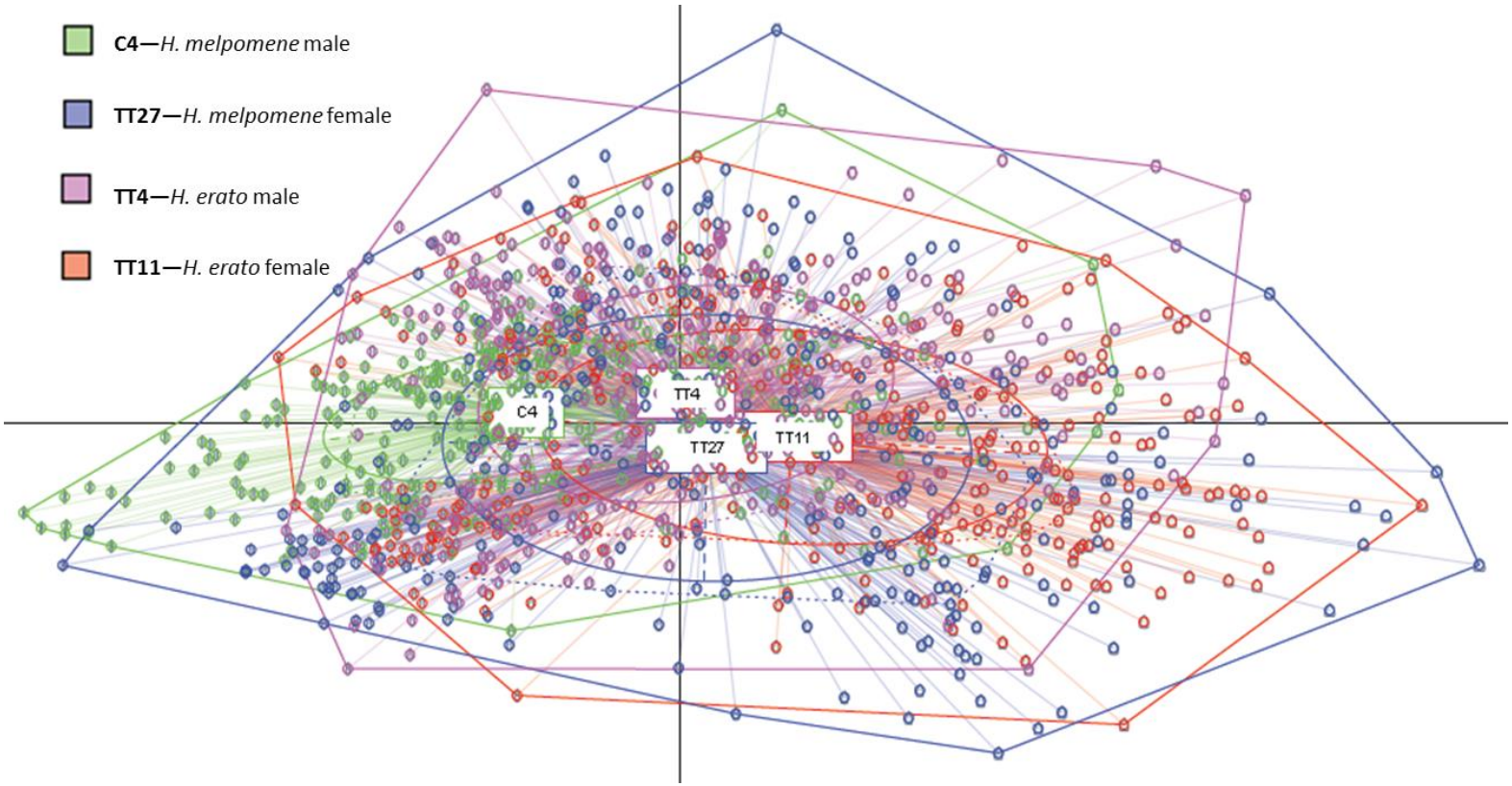

Figure 29.) Convex hull graph of the PC1/PC2 morphospace data. The irregular colored lines around the outside of the data points show the distribution of the scale shapes. The ellipses near the center show the averages. This method of visualizing the data represents similar trends to those shown in Figure 28, with the females of both species closely associated and located near the bottom of the morphospace. The two males seem to overlap more in this graph, but both are still concentrated more in the upper quadrants of the morphospace than the females.

In the upper right quadrant, where the scales are mainly ovular, the two cells of the hindwing, C and Sc+R1, contribute the most to the scale shape variation. The 2A and CuA2 cells of the forewing are concentrated in both the lower right sections and throughout the center of the morphospace, with morphologies ranging from rounded triangles to thin oblongs. Along the far left margin of the morphospace, the trend of scale shape distribution by wing breaks down. Here, where the scale morphologies are thin, narrow oblongs, the main contributors are the 2A cell of the forewing and the C cell of the hindwing. Both of these are marginal cells, meaning they run along the long edge of the wing (the posterior edge of the forewing and the anterior edge of the hindwing), and are where acute scale types are found, which can have the thin, pointed morphology represented in the morphospace.

Discussion:

Through the morphological analysis, some interesting trends emerged that were not captured by the scale count approach. Since the grouping of scales into general shape categories misses variation within the scale type, and is a somewhat subjective method of analyzing, very little difference between specimens or wings was detected. Some dissimilarity could be distinguished in the distribution of the scale type percentages in each sampling site, but not in the actual scale shapes. From my own observations, it was a little surprising that each specimen differed from the others. Even after imaging and counting thousands of scales from each

butterfly, I do not think that I could accurately identify the species of a specimen by examining the scales alone, and could only determine sex if androconia were present. This implies that the analysis was sensitive enough to capture variation in scale shape that is difficult to distinguish with the human eye alone.

Even more intriguing is the clear distinctions between fore and hindwing in the PC1/PC2 morphospace. Before examining this data, I would have predicted that the scale shapes would be more likely to group by cell type (interior or marginal), rather than wing. From my scale count data, the distributions of scale types are much more similar between the interior cells of both wings (CuA2 and Sc+R1), and the between the marginal cells found along the long edge of the wings (2A and C). The PC1/PC2 morphospace coded by cell, however, shows that fine scale shape is determined more by forewing or hindwing than by cell. The exception to this, which seems to uphold my observations and scale counts, is the close association between the 2A cell of the forewing and the C cell of the hindwing. These two marginal cells are grouped together at the far left edge of the morphospace, where the scale shapes seem to be narrow, somewhat pointed oblongs. Acute scales, which are only found in the marginal cells, can sometimes be found in this shape. That both marginal cells seem to contribute to the variance of this acute scale morphology concurs very well with the scale count data.

The statistically analyzed morphology data supports different aspects of the more qualitative scale count data, though with complications. When the scale shapes were compared between wing cells and cell regions, the results were

extremely significant. The trends I saw represented in the scale count data can draw support from this, as both wing cell and location on the wing seemed to be important factors in determining scale shape. Though the differences between individual specimens were more difficult to determine by counting scale types, there did seem to be some variations in the strength and location of shape distribution changes. The statistical analysis presented similar results, with scale shape differing significantly between the two species, and between the two sexes of each species.

While the statistical support from the scale morphology analysis does give some mathematical credence to the qualitative trends I observed, the fact that everything is significant indicates the need for controls in future studies. If every specimen, every cell and every cell region is significantly different, it could mean that scale shape is so variable that it has no real biological importance. This possibility points to the necessity of a control. Would two males of the same species, sampled in the same way I sampled these butterflies, come out similar to each other, or significantly different? If I could get twice as many scales from each sampling position on one butterfly and then randomly assigned them to two groups, would these groups come out the same, or significantly different? In the case of this study, I would be reluctant to say that the ubiquitous, significant differences indicate insignificance. Four specimens are too few to concretely describe a trend for the rest of their species. From my observation of scales under the microscope, it could also be that specific scale shape is simply very variable.

Within the general categories that I grouped scales, I saw large amounts of diversity that could have been captured by the statistical analysis. The number of tines, the symmetry of the scale, and the shape of the base can all differ drastically within one type. Scales are formed from a single cell, which means that they have few correctional powers for developmental aberrations, and perhaps some tolerance for individual vagaries that have little to no consequence on their effectiveness on the wing.

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