Do butterflies use “hearing aids”? Investigating the structure and function of inflated wing veins in Nymphalidae

by

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Abstract

Many butterfly species within the subfamily Satyrinae (Nymphalidae) have been informally reported to possess a conspicuous “inflated” or “swollen” subcostal vein on each forewing. However, the function and taxonomic diversity of these structures is unknown. This thesis comprises both experimental and comparative approaches to test hypotheses on the function and evolution of these inflated veins. A laser vibrometry study showed that ears in the common wood nymph, *Cercyonis pegala,* are tuned to sounds between 1-5 kHz and the inflated subcostal vein enhances sensitivity to these sounds. A comparative study showed that all species with inflated veins possess ears, but not all species with ears possess inflated veins. Further, inflated veins were better developed in smaller butterflies. This thesis provides the first evidence for the function of inflated wing veins in butterflies and supports the hypothesis that they function as aids to low frequency hearing.

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Lastly, I thank anyone who reads this in the future, for any reason.

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Chapter 1. General Introduction

1.1. Acoustic communication in insects

Insects are highly acoustic, using sounds and vibrations in a variety of contexts, including predator detection and deterrence, intraspecific communication, finding food, and orientation (Yack & Dawson, 2008; Mason & Pollack, 2016). Insects have diverse ways of generating and sensing sounds and vibrations. One of these ways involves tympanal organs, a type of mechanoreceptor used to detect airborne sounds (Yack, 2004; Yack & Dawson, 2008). A tympanate ear is characterized by three main features: (1) a tympanum or ear drum, generally an ovular or rounded membrane of varying thickness, which vibrates in response to sound; (2) an air-filled tracheal sac/space, which forms a reverberation chamber over which the tympanum is stretched; and (3) the chordotonal organ(s) with underlying scolopidia, which detect the vibration of the membrane and produces a neural response (Yager, 1999; Yack, 2004).

Tympanal ears are the main sensory organs in insects that detect far-field sounds. They are morphologically diverse, resulting in diverse functions as well. Selection pressure from factors such as the environment, predators, and mating systems, has influenced the variety of placements, designs, and capabilities of hearing systems (Stumpner & Helversen, 2001; Mason & Pollack, 2016). In different insect taxa, hearing organs can occur on almost any part of the body, including the mouthparts, wings, and legs, and vary considerably in their structure, function, and complexity (Yack, 2004). Some insects also have specialized accessory structures associated with their hearing organs that further provide them with a selective advantage or compensate for the physical challenges of hearing that are associated with a small body size, such as acting

as a resonator, amplifying certain frequencies, or providing directional information (Michelsen et al. 1994; Bennet-Clark, 1999; Schmidt & Romer, 2013; Hirtenlehner et al. 2014). While the function, mechanics, and accessory structures involved with hearing in some insects, such as moths, grasshoppers, cicadas, and crickets, have been well-studied (e.g. Minet & Surlykke, 2003; Balakrishnan, 2016; Greenfield, 2016), we know relatively less about tympanal organs and accessory structures in other taxa, such as butterflies. This thesis will focus on hearing in butterflies, and specifically will examine accessory structures that may act as "hearing aids."

1.2. Butterfly ears

1.2.1. Acoustic communication in butterflies

Relatively little is known about acoustic communication among butterflies, especially diurnal butterflies, compared to the diversity of information established for butterfly visual and chemical sensory ecology (Eltringham, 1919; Ford, 1945; Silberglied, 1977; Ackery et al. 1998). Although sound production that functions in courtship or defense has been reported for a variety of moth families (Spangler et al. 1984; Greenfield, 2014; Greenfield, 2016), sound production is rarely reported from butterflies (Greenfield, 2016). Some species of butterflies are known to make and use sounds, such as clicking with their wings to communicate with conspecifics (Otero, 1990; Yack et al. 2000), or making ultrasonic clicks to repel predators like bats (Mohl & Miller, 1976).

Although it is established that few butterflies perform sound production, many mute species can still hear sounds. Butterflies comprise 3 superfamilies of the order Lepidoptera, which also includes 43 superfamilies of moths. These superfamilies are: Papilionoidea, Hesperoiidea, and Hedyloidea. Tympanal hearing in moths has been studied extensively, particularly in the superfamilies Noctuoidea, Geometroidea, and Pyraloidea (Minet & Surlykke, 1998; Yack, 2004). Moth ears occur on different parts of the body but mostly on the thorax and abdomen, and function primarily to detect the echolocation calls of insectivorous bats (Minet & Surlykke, 1998; Yack, 2004; Greenfield, 2016). Relatively less is known about hearing in butterflies.

1.2.2. Hearing in butterflies

Hearing in butterflies has been reported in 2 main taxa – the Hedylidae and the Nymphalidae (Swihart, 1967; Yack & Fullard, 2000; Yack et al. 2007). Hedylidae butterflies, representing the superfamily Hedyloidea, are nocturnal and possess ultra-sound sensitive tympanate ears (>20 kHz). Among diurnal butterflies, Nymphalidae, of the superfamily Papilionoidea, is the only family reported to date to include species that have ears. Nymphalidae is one of the largest families of Lepidoptera, comprising 12 subfamilies, about 400 genera and 6000 species occurring worldwide (Ackery et al. 1998; Wahlberg et al. 2009). Ears in Nymphalidae are called Vogel's organs (le Cerf, 1926; Minet & Surlykke, 2003; Yack & Dawson, 2008). Vogel's organ is located on the ventral surface of the wings, at the base of the forewing subcostal and cubital veins, and consists of the tympanic membrane, the tympanum, bordered by a chitinous ring, and is associated with 3 chordotonal sensory organs and enlarged tracheae in the species that have been

studied to date (Vogel, 1912; Yack et al. 2000; Lane et al. 2008; Lucas et al. 2009; Lucas et al. 2014).

1.3. "Inflated" wing veins

1.3.1. What are they?

Some butterflies possess conspicuously enlarged or "inflated" wing veins, but prior to this thesis, this characteristic lacked quantitative assessment. The wing veins of some species, as in the subfamily Satyrinae, have been described as inflated, thick, or swollen (Ford, 1945; Devries, 1987; Monge-Najera & Hernandez, 1991). In other studies, it has been used as a taxonomic character (Ford, 1945; Ackery et al. 1998), but only qualitatively described without quantification, such as a "rounded protuberance of the vein" (le Cerf, 1926), a "very thick area in the subcostal vein of the forewings" (Devries, 1987), "thickened veins that close the discal cell at the apex" (Otero, 1990), and in *Hamadryas* butterflies, "highly swollen, reaching a diameter of 2-3 times that of the equivalent vein of silent species" (Monge-Najera & Hernandez, 1991). Murillo-Hiller (2006) even describes in *Euptychoides castrensis* (Satyrinae: Satyrini) that the "widest part of the swollen area in their subcostal vein is 800 μm, about eight times the 100 μm width of the vein's base, and the length of the swollen area is 6000 μm, 1/3 of the distance to the distal wing border". Despite these descriptions provided for some species, this trait has never been formally quantified, nor has it been studied for its functional significance. Some inflated veins also are proposed to have internal structures that form geometric configurations. Monge-Najera et al. (1998) and Murillo-Hiller (2011) describe that the swollen veins in several species of *Hamadryas* have a "serpentine" or spiral

structure inside. Murillo-Hiller (2006) also found that the swollen area of the subcostal cell of *E. castrensis* contained many small irregular polygonal chambers of membranous walls, with sides measuring around 200 μm, and with varying volumes. Overall, descriptions of these inflated veins and their internal structures are rare in the literature and have not been formally characterized.

1.3.2. Who has inflated wing veins?

Inflated veins have only been noted to occur in some species belonging to 3 subfamilies to date: Satyrinae, Biblidinae, and Charaxinae, all within the family Nymphalidae (Ford, 1945; Miller, 1968; Jenkins, 1983; Ackery et al.1998; Monge-Najera et al. 1998; Freitas & Brown, 2004). They were previously used as an informal distinguishing characteristic of the subfamily Satyrinae, which previously excluded tribes Morphini and Brassolini (Ford, 1945; Miller, 1968; Ackery et al.1998). This character continues to be used as a distinguishing feature of different taxa (e.g. Freitas and Brown, 2004). Table 1 provides information on what that has been done and proposed to date before the research undertaken in this thesis. Because the character has not been formally characterized, it remains unclear how this character is taxonomically distributed.

1.3.3. What is/are the function(s) of inflated veins?

The function of wing vein inflations has not been experimentally tested until the research undertaken for this thesis, although a few hypotheses have been proposed. One hypothesis is that the inflated veins are used for sound production in sound producing

butterflies, such as in the genus *Hamadryas* (Otero, 1990; Monge-Najera & Hernandez, 1991; Monge-Najera et al. 1998; Murillo-Hiller, 2006; Marini-Filho & Benson, 2010). Table 1 provides information on whether the veins have been noted to be used in sound production. For example, Otero (1990) suggests that *Hamadryas feronia* (Biblidinae: Biblidini) produces percussive sound through the striking of the wings, and the structures responsible for the loud snapping sound are the swollen veins, and Monge-Najera and Hernandez (1991) suggest that the swollen base of the subcostal vein in the forewings in noise-producing *Hamadryas* species could be a reinforcement for percussion through the striking of the wings. Murillo-Hiller (2006) suggests that the internal structure chambers inside the swollen veins of *Euptychoides castrensis* (Satyrinae: Satyrini) amplify the sound created when it is struck from the wing. However, these studies did not provide experimental evidence, and further studies made by Yack et al. (2000) in *Hamadryas feronia* contradict the percussion mechanism arguing that a single forewing can produce sound. Furthermore, most of the species noted for wing vein inflation (see Table 1) have not been reported to produce sounds and are assumed to be mute until further evidence. Thus, evidence for this hypothesis remains unconvincing or at least inconclusive at present. Therefore, it is important to consider alternative hypotheses that explain the function(s) of inflated veins.

A second hypothesis that may explain the function of inflated wing veins is that they enhance or aid in flight. Wing vein morphology has been shown in several insects to affect flight dynamics (Wang et al. 2008; Tanaka & Simoyama, 2010), but the role of inflated veins has not been proposed to function in flight in butterflies or any other insect.

Although this may be worth testing in the future. I will not be pursuing this idea in my study.

The third and most plausible hypothesis is that inflated veins are associated with hearing. If so, first, a connection between the Vogel's organ (the ear) and the inflated vein is expected. Some morphological studies have shown that they may be linked; le Cerf (1926) illustrates and notes that the cuticle of the VO occurs at the end of the rounded protuberance of the vein, and Monge-Najera et al. (1998) uses morphological methods to suggest that the cubital vein is connected to the VO. Vogel's organ works to help with hearing because there is an air space behind the membrane that allows the membrane to vibrate and stimulate the chordotonal organs for hearing. A second prediction supporting the hypothesis that inflated veins are associated with hearing is that they need to occur in species with Vogel's organs, and not in species lacking Vogel's organ. So far, every study that has mentioned inclusion of a swollen vein involves a species that also has an ear (le Cerf, 1926; Monge-Najera & Hernandez, 1991; Monge-Najera et al. 1998; Yack et al. 2000; Chapter 3), though this remains to be formally tested. Finally, if inflated veins are associated with hearing, it would be predicted that ablating the vein affects hearing by way of tuning properties of the ear. This has also yet to be formally tested. Thus, given these predictions for both hearing and size hypothesis, my thesis will focus on testing this third and most compelling function for inflated veins.

Table 1.1. Previous literature about vein inflations

Family: Subfamily	*Species*	*Mention of swollen veins (Forewing)*[1]				*Possible sound production (Y/N)*	*Comments*	*Reference(s)*
		Costal (Y/N)	*Subcostal (Y/N)*	*Cubital (Y/N)*	*Anal (Y/N)*			
Nymphalidae: Satyrinae	Swollen veins are a distinguishing or key feature of the entire family group	N	Y	Y	Y	N	Swollen veins at the base of the forewing is a distinguishing or key feature. ("one or more" – Ford, 1945) ("the three basic forewing veins" – Miller, 1968) ("some of the veins" – Comstock, 1949)	Ackery et al. 1998; Comstock, 1949; Ford, 1945; Miller, 1968
Nymphalidae: Satyrinae	*Euptychia terrestris* *Euptychoides castrensis*	N	Y	N	N	Y (*E. castrensis* by Murillo-Hiller, 2006)	Widest part of the swollen area is 800 µm, about eight times the 100 µm width of the vein's base; is proposed to be used for sound production (see *1.3.3. what are their functions)*	Le Cerf, 1926; Murillo-Hiller; 2006
Nymphalidae: Biblidinae	Generally, butterflies of genus *Hamadryas*	N	Y	Y	N	Y	Characterized as "swollen subcostal vein" and "adults have swelling of the bases or lower half of the subcostal and cubital veins"	Jenkins, 1983; Monge-Najera et al. 1998
Nymphalidae: Biblidinae	*Hamadryas: H. amphinome, H. feronia, H. guatemalena, H. glauconome, and H. februa*	N	Y	N	N	Y	They characterize swollen veins as the "swollen base of the subcostal cell."	Monge-Najera & Hernandez (1991); Yack et al. 2000
Nymphalidae: Biinae, now part of Charaxinae	Species within *Biinae*	Y	N	N	N	N	"inflated costal veins" (Biinae was a subfamily, but now is a genus named *Bia*)	Freitas & Brown, 2004

[1] At present, no literature has referred to inflated veins occurring in any hindwing veins or forewing radial veins

1.4. Thesis overview

The overall goal of this thesis is to test the hypothesis that enlarged wing veins function as "hearing aids" in butterflies. The sub-goals of this thesis are: (1) Review the literature summarizing current knowledge on butterfly ears and hearing, and the inflated veins, including their function and distribution (Chapters 1, 2, 3); (2) Characterize the ear and inflated vein of a model species with this trait, the Common Wood Nymph, *Cercyonis pegala*, and experimentally test and explain its function in relation to hearing, and (3) Formally characterize and quantify the inflated vein and assess its function and relationship to hearing and body size.

The first hypothesis tested is the "hearing aid" hypothesis: inflated veins are associated with hearing. It is predicted that (a) inflated veins are connected with the ear of the butterfly, Vogel's organ (Chapter 2), (b) ablating the wing vein impacts the of the ear's sensitivity or tuning properties (Chapter 2), and (c) inflated veins occur primarily in species that have ears (Chapter 3). The second hypothesis tested is the "size" hypothesis: inflated veins are more pronounced in smaller species. This hypothesis is based on the fact that small insects face difficulty in hearing due to physical constraints. It is predicted that (a) inflated veins are more developed in smaller species, and that the degree of inflation is negatively correlated with body size (Chapter 3), and (b) species with more than one enlarged vein will be relatively small (Chapter 3). The information gained from this research will be summarized, and suggestions for guiding future studies on butterfly sensory ecology, butterfly systematics, and applications of novel acoustic technologies will be discussed (Chapter 4).

Chapter 2. Butterflies use Inflated Wing Veins as "Hearing Aids"

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Contribution statement

Penghui Sun collected specimens, conducted laser vibrometry trials, data analysis, and co-drafted the manuscript. Natasha Mhatre coordinated and conducted laser vibrometry trials and advised on data interpretation and analysis. Andrew Mason advised on laser vibrometry trials and data interpretation and analysis. Jayne Yack coordinated the study, contributed to experimental design, collected specimens, and co-drafted the manuscript. All authors contributed to editing and gave approval for manuscript submission.

2.1. Abstract

Insects have evolved a diversity of hearing organs specialized to detect sounds critical for survival. We report on a unique structure on butterfly wings that enhances hearing. The Satyrini are a diverse group of butterflies occurring throughout the world. One of their distinguishing features is a conspicuous swelling of their forewing vein, but the functional significance of this structure is unknown. Here we show that wing vein inflations function in hearing. Using the Common Wood-Nymph, *Cercyonis pegala*, as a model, we show that (1) these butterflies have ears on their forewings that are most sensitive to low frequency sounds (<5 kHz); (2) inflated wing veins are directly connected to the ears; (3) when vein inflations are ablated, sensitivity to low frequency sounds is impaired. We propose that these miniature 'hearing aids' contribute to low frequency hearing by impedance matching.

2.2. Introduction

Insects have a rich diversity of hearing organs that function in a variety of tasks including locating mates, evading predators, and coordinating social interactions (Yager, 1999). To achieve these tasks, insects need to evaluate the amplitude, frequency, and temporal patterns of sounds (Mason & Pollack, 2016). The small size of insects presents challenges to achieving these tasks and consequently, insect ears have evolved unique specializations (Windmill & Jackson, 2016). Here we investigate how inflated wing veins in butterflies function to enhance hearing.

Satyrinae are a large subfamily of ~2,400 butterfly species belonging to the family Nymphalidae. Many Satyrinae possess ears at the base of their forewings (Vogel, 1912; le Cerf, 1926; Minet & Surlykke, 2003), but little is known about the characteristics of their hearing. A distinguishing feature of Satyrinae, particularly within the tribe Satyrini, is a conspicuous "inflated", "dilated", or "swollen" vein on each forewing (Ford, 1945; Miller, 1968; Ackery et al. 1998). However, the function of these prominent structures is unknown. We propose that they function in hearing based on their morphological proximity to the ear. Using a model Satyrini, the Common Wood Nymph (*Cercyonis pegala*), we first describe the morphology of the eardrum and characterize its vibration properties. We then test the hypothesis that swollen wing veins function in hearing – specifically in the tuning and sensitivity of the eardrum's response to sound. We predict that: (i) there is a physical connection between the ear and the swollen vein; (ii) ablating the swollen vein will impair hearing.

2.2. Methods

2.2.1. Animals

Cercyonis pegala butterflies were collected from their natural habitat near Ottawa and Perth, Ontario, Canada between July and August 2016 and 2017 (Appendix 1). Specimens used for laser vibrometry were stored in glassine envelopes at ~8°C for up to 6 days prior to conducting experiments. A total of 30 specimens were used; seven males and seven females for laser vibrometry and all 30 for morphology.

2.2.2. Tympanal and wing vein morphology

Tympanal membrane and forewing vein morphology was examined in 15 males and 15 females. Photographs were taken using a light microscope (Leica M205C) equipped with a camera (Leica DMC4500) and tympanal surface area was measured (Leica Application Suite 4.8.0). Scanning electron micrographs (SEM) were used to image the tympanal membrane and the connectivity between the tympanal chamber and subcostal vein.

2.2.3. Laser vibrometry and acoustic stimuli

Vibration measurements were made with a scanning laser Doppler vibrometer (Polytec PSV 400) coupled with an OFV-505 sensor fitted with a close-up unit. Anesthetized butterflies were mounted on a rotatable metal platform attached to a steel rod. The forewing and abdomen were immobilized, and wing-scales removed to expose the tympanum. For each butterfly, the entire ear membrane was scanned, approximately 150-200 measurement points on a grid (Figure 2.2a). Vibrations of the tympanic membrane were measured in response to acoustic stimuli: 160 ms periodic chirps from 0.75 to 20 kHz with a frequency resolution of 6.25 Hz (Appendix 1). The sound pressure level (SPL) was measured using a calibrated microphone (Bruel and Kjær, Microphone: 4138) coupled to a pre-amplifier (Nexus: 2690). Both acoustic and vibrational data were digitized at a sampling rate of 51.2 kHz. The magnitude, phase, and coherence of each membrane's displacement was plotted as a frequency response at each scan point which was calculated from measurements of the membrane's velocity at each point (Appendix

1). Video animations of the membrane vibration were created using the PSV software (Appendix 1).

The vibration properties of the tympanal membrane with the vein intact were measured for each individual. Following the initial scan, the inflated subcostal vein ipsilateral to the ear was ablated by making a longitudinal cut to open the ventral surface (Appendix 1: Figure A1.2), and the vibration pattern of the same tympanum was remeasured.

2.2.4. Data analysis

Comparison of tympanal vibration characteristics between sexes was done using two-sample t-tests with unequal variances. The magnitude of the membrane transfer function (at point of highest displacement) was plotted using Matlab R2017b (v9.3.0.713579) and fitted to a damped simple harmonic oscillator (SHO) model. Parameters for a SHO, such as resonance frequency, were estimated and fitted using Matlab's Curve Fitting toolbox, and corresponding displacements at resonance frequency were calculated from the fitted models. Mean resonance frequency and mean displacement at resonance frequency, were compared between the intact and ablated conditions using paired two-sample t-tests.

2.3. Results

2.3.1. Morphology

Cercyonis pegala has a well-developed tympanal ear located at the base of the ventral forewing (Figure 2.1a). The membrane is oval-shaped and is bordered by a chitinous ring (Figure 2.1b, c). Both males and females have well-developed tympanal membranes, with surface areas of .243 ± .040 and .274 ± .032 mm^2 respectively. Surface areas did not differ between sexes despite differences in body size (Appendix 1: Table A1.1). The subcostal vein is visibly enlarged (Figure 2.1, Appendix 1: Figure A1.3) and physical connected to the tympanal chamber (air space beneath the tympanum) (Figure 2.1f). The enlarged subcostal vein contains an internal network of epithelial tissue that forms a honeycomb-like configuration (Figure 2.1c-e).

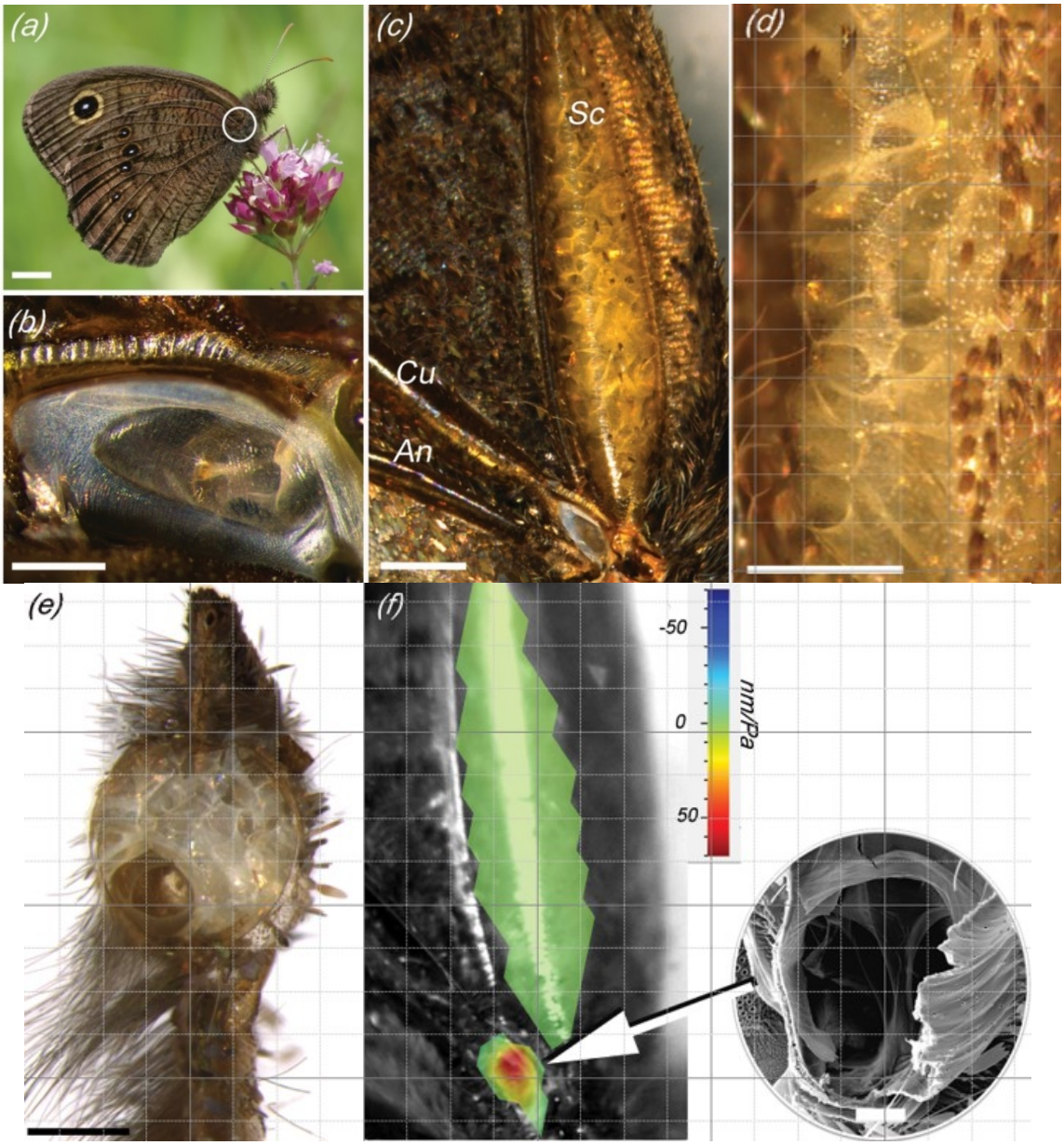

Figure 2.1. Ear and wing vein morphology of C. pegala. (a) Butterfly in resting position. A white circle marks the location of the ear. Scale bar: 5 mm; (b) Light micrograph of right tympanal membrane. Scale bar: 200 µm; (c) Forewing showing enlarged subcostal (Sc) vein, as well as cubital (Cu) and anal (An) veins. Tympanal ear is seen at the wing base. Scale bar: 1 mm; (d) Internal structure of Sc vein viewed through the cuticle. Scale bar: 500 µm. (e) Cross section of the Sc vein. Scale bar: 500 µm. (f) Laser scan of Sc vein and tympanal membrane depicting displacement at 4.8 kHz. Inset: Scanning electron micrograph of the opening connecting the tympanal chamber and Sc vein. Scale bar of inset: 100 µm.

2.3.2. Mechanical response of tympanum

The mechanical response of the eardrum in *C. pegala* was measured in 7 males and 7 females with intact veins (Figures 2.1f, 2.2, Table 2.1). There were no significant differences between sexes (Appendix 1: Table A1.1, Figure A1.4). The tympanum is most responsive to low frequency sounds (<5 kHz) and behaves like a low-pass filter. The response is flat below 5 kHz and decreases between 5-8 kHz, before falling steeply beyond 8 kHz (Figure 2.2c). Mean maximum displacement is 182 $\pm$ 102 nm/Pa and frequency at maximum displacement is 4.39 ± 3.01 kHz (Figure 2.2b, Table 2.1). The phase change is very gradual around the resonance frequency (7.8 ± 1.2 kHz), which is itself higher than the frequency of maximal displacement. Taken together, this indicates that the *C. pegala* ear is a highly damped and non-resonant system, which is adapted to respond equally to a broad range of low frequency sounds.

When the subcostal vein is ablated, the membrane shows reduced sensitivity overall (Appendix 1: Movie A1.1), but particularly to sounds at the lower end of this frequency range (0.75-5 kHz) (Figure 2.2c, Table 2.1). The response amplitude is no longer flat, is erratic for frequencies from 0.5 to 1.5 kHz and then steadily increases, exhibiting a maximum at 5.99 kHz. The mean maximum displacement and the mean displacement of the membrane at resonance frequency were both significantly lower in the ablated condition (Figure 2.2c, Table 2.1), although the mean resonance frequency of the membrane in the ablated condition remained unchanged (Table 2.1). The spatial pattern of response amplitude also remains similar. These results show that ablating the inflated vein does not change the mechanical properties of the membrane yet results in reduced sensitivity to lower frequency sounds. Our data clearly shows that the inflated

vein is connected to and functionally active in the *C. pegala* auditory system. The data also suggests that the vein inflation may be crucial to developing the flat frequency response observed in the *C. pegala* ears. The data also suggests that the vein inflation is crucial to developing the flat frequency response observed in the ears.

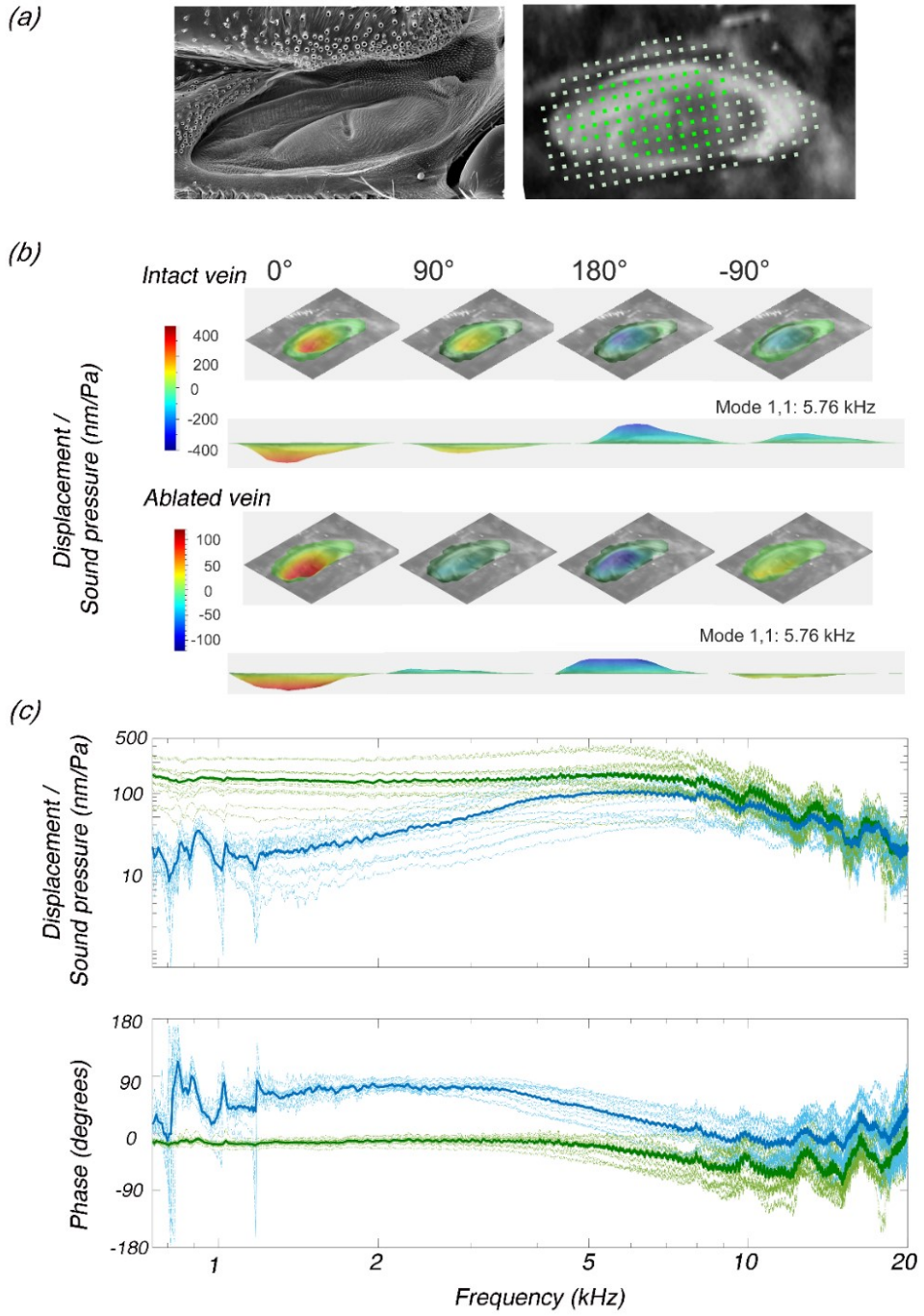

Figure 2.2. Vibrational response of tympanal membrane in *C. pegala*. (a) Scanning electron micrograph of tympanal membrane (left) and laser scanning grid (right). (b) Tympanal displacement per unit sound pressure level in response to 5.7 kHz (resonant frequency) when inflated vein is intact (top) or ablated (bottom). Each is shown at 4 phases of the oscillation. Red: outward deflection, green: inward deflection. Note the difference in scale between the two conditions. (c) Displacement (top) and phase (bottom) of tympanal membrane relative to frequency for intact (green) and ablated (blue) conditions. (N=14, solid lines: mean).

Table 2.1. Tympanal membrane responses in intact and ablated condition. Significant difference between intact and ablated conditions determined using paired Student's t-test for unequal variances ($p<0.01$ taken as significant). n = 7 males, 7 females

Parameter	Intact (n=14)	Ablated (n=14)	p-value
Mean resonance frequency (kHz) (±s.d.)	7.78±1.21	8.08±1.55	t=-1.3450, df=13, p=0.1008
Mean displacement at resonance frequency (nm/Pa) (±s.d.)	156±50.1	98.1±48.0	t=3.5980, df=13, p=0.0016
Mean maximum displacement (nm/Pa) (±s.d.)	204±99.3	122±53.6	t=4.5040, df=13, p=0.0006
Mean frequency at maximum displacement (kHz) (±s.d.)	4.39±3.01	7.64±3.36	t=-2.5801, df=13, p=0.0228

2.4. Discussion

Tympanal ears were first described morphologically in Satyrini butterflies more than 100 years ago (Vogel, 1912), but until now, the tuning characteristics of these ears had not been studied. Our results from a diurnal Satyrini species, *C. pegala*, show that the tympanal membrane is broadly tuned to low frequency sounds (<7 kHz). Sensitivity to low frequency sounds concurs with one behavioural study of Satyrini species *Erebia euryale* and *E. manto* (125 Hz - 16 kHz) (Ribaric & Gogala, 1996), and neurophysiological studies of non-Satyrini species, *Caligo eurilochus* (1-4 kHz) and *Morpho peleides* (1-5 kHz) (Lucas et al. 2009; Lucas et al. 2014; Mikhail et al. 2018). The functional significance of hearing in butterflies is not fully understood, but evidence to date indicates that they function to detect broadband sounds of diurnal predators, including bird flight and calls that overlap with hearing sensitivity of butterflies (Ribaric & Gogola, 1996; Fournier et al. 2013; Mikhail et al. 2018).

While our results clearly support the hypothesis that inflated veins function as 'passive' hearing aids by increasing sensitivity to low frequency sounds, a number of

details remain to be resolved. The ear may function either as a pressure or a pressure difference receiver ear, as sound may only reach the tympanal membrane externally, or, may have a yet undiscovered path. Inflated veins may contribute to hearing by acoustic impedance matching, whereby the volume of air provided by the inflated vein behind the ear would allow the ear to respond to low frequencies which would otherwise cancel out from an absence of a gradient (Michelsen & Larsen, 2008). The honeycomb-like structures within the inflated vein may also contribute to enhancing hearing, perhaps functioning in damping the membrane response like honeycomb sandwich panels in buildings to provide noise transmission loss (Wen-Chao & Chung-Fai, 1997). Our results show that inflated veins provide butterflies with a unique mechanism of auditory frequency tuning, with unusually 'flat' frequency responses that may have implications for novel acoustic technology. How these veins contribute to such specialized tuning will be further explored by modeling, and experimental manipulation of internal structures.

This study resolves a century old conundrum concerning the function of inflated wing veins in butterflies. We show that they function in hearing. Small insects face physical challenges in hearing low frequencies (Bennet-Clark, 1998; Michelsen & Larson, 2008; Windmill & Jackson, 2016). Thus, vein inflations may occur in other smaller species of butterflies. Alternatively, vein inflations may evolve in species that benefit from enhanced low frequency hearing, perhaps due to habitat or predator differences. Hearing in butterflies is widespread, but at present little is known about the function and evolution of these sensory organs. Further experimental and comparative studies are essential to better understand the acoustic sensory ecology of these ecologically important insects.

Chapter 3. Wing Veins as "Hearing Aids" in Butterflies: A Comparative Study

Parts of this chapter are being prepared for publication.

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Contribution statement

Penghui Sun performed all measurements and raw analyses. Akito Kawahara provided specimens. Jayne Yack coordinated the study and collected specimens.

3.1. Abstract

Butterflies possess hearing organs at base of their wings, but little is known about these structures, their taxonomic distribution, and specializations. Within Nymphalidae, species within the subfamily Satyrinae are noted to have a characteristic feature deemed the "inflated vein", a distinctive swelling of their forewing subcostal vein. Chapter 2 of this thesis experimentally supported the hypothesis that these inflations are involved with hearing in the Satyrini species *Cercyonis pegala*. This chapter (Chapter 3) takes a comparative approach to better understand the taxonomic distribution of this trait, and to further test the 'hearing aid' hypothesis and the 'size' hypothesis. Specifically, I studied the relationship between vein inflations and hearing organs and examined whether body size was related to the size of inflated veins. Species among various subfamilies within Nymphalidae were sampled, including 52 Satyrinae species, representing all tribes and a variety of body sizes. To formally quantify the 'degree' of the Subcostal (Sc) vein inflation, two measures were used: 1. the ratio between the widest regions of the forewing subcostal vein and the hindwing subcostal vein; 2. the ratio between the widest region of the forewing subcostal vein and the widest region of the forewing radial vein just before it splits (intersection of R1 and R2) to represent a reference vein width. There was a wide variation between species in the degree of Sc vein inflation, regardless of ratio method. First, the results show that all species with large vein inflations also have tympanal ears, supporting the hypothesis that inflated veins function in hearing. Not all species with ears however have inflated veins, showing that inflated veins are not necessary for hearing in all species. Second, the results show that large Sc vein inflations are most prominent in Satyrinae, particularly in the tribe Satyrini, supporting previous literature distinguishing

this trait as a characteristic of the group. To explain variation in the degree of vein inflation between species, I tested the "size" hypothesis, that inflated veins are more prominent in smaller than larger species. Preliminary analyses, without accounting for phylogeny, show a strong negative correlation with body size: inflated veins are larger in smaller species, as predicted. This result provides preliminary support for the hypothesis that vein inflations evolved in smaller butterfly species. I discuss how there is selection pressure on small insects to evolve hearing specializations as they face physical challenges in hearing low frequency sounds. This is the first study to formally quantify wing vein inflations in butterflies, to study how this trait varies between species, and to seek an explanation for this variation.

3.2. Introduction

Insects are highly acoustic, using hearing for purposes of survival and reproduction, such as predator detection, intraspecific communication, or host localization (Mason & Pollack, 2016; Pollack, 2016). They have evolved a wide diversity of hearing structures specialized to detect and localize sounds, such as specializations for tuning or amplifying specific frequencies, providing detailed directionality about environmental cues, or overcoming physical restraints in being able to hear sounds, particularly lower frequencies (Bennet-Clark, 1999; Stumpner & Helversen, 2001; Strauß & Stumpner, 2014). Hearing specializations have been well studied in a few selected taxa, including crickets and their host, *Ormia* flies. Crickets have evolved complex and modified systems of trachea to enhance sound conduction and help with sound directionality (Schmidt & Romer, 2013), as well as active amplification techniques to

enable high auditory sensitivity and tuning to conspecific songs (Mhatre & Robert, 2013). *Ormia* flies have a flexible bridge-like structure between its two ears that bends to localize and amplify sound vibrations (Robert et al. 1994, 1996). Other taxa, such as butterflies, have well-developed ears, but little is known about their hearing, and much less about any hearing specializations. This chapter will focus on inflated wing inflations as possible hearing specializations in Nymphalidae butterflies.

3.2.1. Hearing in butterflies

Butterflies belong to 3 superfamilies (Papilionoidea, Hedyloidea and Hesperoidea) of the insect order Lepidoptera, which also includes 43 superfamilies of moths. Butterflies serve as bioindicators, pollinators, and even symbols of beauty and importance for people (Brown & Freitas, 2000; Liu, 2001; Nabhan et al. 2006; Gehring & Bennett, 2009; de Araújo et al. 2014). Due to their ecological and economic importance, understanding butterfly sensory ecology is paramount to contributing to the knowledge and preservation of our world. They have been well studied for their visual and chemical sensory ecology, but relatively less is known about their sense of hearing. Hearing in butterflies has been reported in 2 main taxa – the Hedylidae and the Nymphalidae (Yack & Fullard, 2000; Minet & Surlykke 2003; Yack et al. 2007).

Hedylidae butterflies, representing the superfamily Hedyloidea, are nocturnal and possess ultra-sound sensitive tympanal ears (>20kHz) at the base of their forewing (Yack & Fullard, 2000; Yack et al. 2007). When these butterflies are exposed to ultrasound, they exhibit a variety of evasive flight maneuvers, including random changes in direction and increased velocity. This is presumed to be an adaptive behaviour to detect and evade

their bat predators (Yack & Fullard, 2000). Neurophysiology trials, recordings from nerve IIN1c, confirmed sensitivity to ultrasonic sounds, with broadband tuning to frequencies between 40 and 80 kHz, but not low frequency (<10 kHz) sounds (Yack et al. 2007).

Nymphalidae, of the superfamily Papilionoidea, is one of the largest families of Lepidoptera, comprising 12 subfamilies, about 400 genera and 6000 species occurring worldwide (Ackery et al. 1998; Wahlberg et al. 2009). Species within the subfamilies Apaturinae, Charaxinae, Biblidinae, Satyrinae, and Calinaginae have been noted to possess a well-developed tympanal ear (Vogel's organ (VO)) based on external morphology (Otero, 1990; Freitas & Brown, 2009; Lane et al. 2008). This structure has been confirmed to function as an ear in several species to date, based on neurophysiology and behaviour, such as in *Hamadryas feronia* (Yack et al. 2000), *Morpho peleides* (Lane et al. 2008; Lucas et al. 2009; Mikhail et al. 2018), *Caligo eurilochus* (Lucas et al. 2014), and *Pararge aegeria* (Mahony, 2006). Vogel's organ is located on the ventral surface of the forewings, at the base of the subcostal and cubital veins, and consists of the tympanic membrane (tympanum), bordered by a chitinous ring, and is associated with 3 chordotonal sensory organs and enlarged tracheae in the species that have been studied to date (Vogel, 1912; Yack et al. 2000; Lane et al. 2008; Lucas et al. 2009; Lucas et al. 2014). Ears demonstrate a wide variation in external morphology. Tympanal membranes vary in colour, thickness, opacity, and structure, ranging from being flat and apparently homogenous to exhibiting a dome-like membrane called a tholus (Otero, 1990; Minet & Surlykke, 2003; Mahony, 2006; Lane et al. 2008; Preston, 2013; Hall, 2014).

At present, we still know relatively little about the comparative morphology, tuning properties, or function of these ears in most species, though some experimental testing has been performed for a few limited species. Mechanical and/or neurophysiological tuning has determined that *Hamadryas feronia* (Ageroniini: Biblidinae), *Morpho peleides* (Morphini: Satyrinae), and *Caligo eurilochus* (Brassolini: Satyrinae) hear low sound frequencies (<4 kHz) (Yack et al. 2000; Lane et al. 2008; Lucas et al. 2009, 2014; Mikhail et al. 2018). Neurophysiologically, ears in *Pararge aegeria* (Satyrini: Satyrinae) were found to respond to sound frequencies between 3 and 18 kHz with a best threshold of 56 dB SPL at 6.5 kHz (Mahony, 2006). The functional significance of low frequency hearing in butterflies is proposed to function in detecting terrestrial or avian predators. Insectivorous bird flight patterns have been characterized to be broadband, low frequency sounds (approximately 20 to 900 Hz) that overlap with hearing in insects (see Fournier et al. 2014). *Erebia manto* and *Erebia euryale* (Satyrini: Satyrinae) also perform escape behaviours in response to sound stimuli at frequencies from 125 Hz to 16 kHz, including opening and closing the wings, wing movement and twitching, fluttering around, and escape flight (Ribaric & Gogola, 1996). While much remains to be learned about the taxonomic distribution and function of hearing in butterflies, accumulating evidence shows that ears are widespread in Nymphalidae, that they vary in their structure, and that they are sensitive primarily to low frequency sounds (i.e. below 8 kHz). This chapter focuses on hearing morphology, and particularly on specialized wing veins and their proposed relationship to hearing in Nymphalidae.

3.2.2. Inflated wing veins

Some butterfly species have been determined to have characteristically unusual enlarged wing veins at the base of their ventral forewing, deemed “inflated veins”. These veins have been used historically as a distinguishing characteristic of the subfamily Satyrinae, described as being “swollen” (Comstock, 1949; Ackery et al. 1998), “dilated” (Ford, 1945), “enlarged” (Scott, 1986), or an “inflation” (Miller, 1968), but little is known about their morphology, and their function has remained untested or inconclusive. Further, using these veins as a distinguishing characteristic for Satyrinae has been confounded and outdated with reorganization of phylogeny and taxonomic groups. For example, earlier references that use the inflated veins as a distinguishing feature of this group refers to Satyrinae (Ford, 1945; Ackery et al. 1998), but implying without inclusion of the tribes Morphini and Brassolini, that were once treated as their own subfamilies and later absorbed within Satyrinae (Pena et al. 2006; Wahlberg et al. 2009). Miller (1968) referred to the group as a family Satyridae, not yet a subfamily. It may be assumed that these older references refer to the group that is now known as the tribe Satyrini when they describe “Satyrinae” or “Satyridae” as being distinguished by this trait. Regardless of their various usage as characters in the taxonomic literature, inflated veins have never been (1) formally measured or quantified, (2) described with respect to external and internal morphology, and (3) their function has remained untested or inconclusive. It was previously proposed that inflated veins are used for sound production in sound-producing butterflies, such as in the genus *Hamadryas* (Otero, 1990; Monge-Najera & Hernandez, 1991; Monge-Najera et al. 1998; Murillo-Hiller, 2006; Marini-Filho & Benson, 2010). Otero (1990) suggests that *Hamadryas feronia* produces percussive sound through the

striking of the wings. By cutting the subcostal veins on one or both wings (forewing and hindwing), he showed the butterflies could not stridulate, and thus concluded that the veins were responsible for the loud snapping sound. Murillo-Hiller (2006) also proposes that the inflated subcostal veins have a possible role in sound production in *Euptychoides castrensis* (formally *Yphthimoides castrensis*), a Satyrinae butterfly, and that *E. castrensis* has subcostal veins 21 times larger than the veins of some species of genus *Hamadryas*, in a wing that is 3 times smaller. This author proposes that this is the reason *E. castrensis* makes a more effective sound than *Hamadryas*; however, no experimental evidence was presented. However, Yack et al. (2000) contradict the stridulating hypothesis, showing that a single forewing can produce sound, such as in *Hamadryas feronia*. Furthermore, at present, most of the species noted for wing vein inflation have not been reported to produce sounds (Chapter 1: Table 1.1.). Thus, evidence for this hypothesis remains unconvincing or at least inconclusive at present.

Another hypothesis that could explain the function of inflated wing veins is that they enhance or aid in flight. Wing vein morphology has been shown in several insects to affect flight dynamics (dragonflies: Wang et al. 2008; swallowtail butterflies: Tanaka & Simoyama, 2010), but the role of inflated veins has not been proposed to function in flight in butterflies or any other insect.

A third hypothesis, proposed in Chapter 2 of this thesis, is that inflated veins aid in hearing. This has been supported by showing a connection between inflated veins and the hearing organ chamber (le Cerf, 1926; Monge-Najera et al. 1998; Stunden, 2014; Chapter 2) and experimental evidence (Chapter 2). The purpose of this chapter (Chapter 3) is to take a comparative approach to testing the hearing aid hypothesis by looking at

their association with hearing organs. Additionally, I will be describing what the morphological variation of these structures in different taxa, and why there is variation. Specific objectives, hypotheses and predictions are outlined below.

3.2.3. Inflated wing veins relative to ears, taxa, sex, and body size

If wing vein inflations function in hearing, then it is predicted that all those with inflations have ears. To test this, the relationship between the presence/absence of ears and the presence/absence of vein inflations needs to be examined. Ears can be qualitatively assigned as well-developed or well-defined (level 2), uncertain (level 1), or absent (level 0) (Preston, 2013) (see also Methods in this chapter). However, vein inflations are harder to characterize, as it is undetermined what type of morphology renders a vein inflation 'functional'. It is uncertain if the degree of inflation should be quantified as categorical or continuous, and if categorical, what threshold is necessary for it to be functional. As a starting point, I will begin by using 2 measurements to assess relative width of the Sc vein: (1) Forewing vein: hindwing vein ratio (FW: HW), and (2) forewing vein: reference vein ratio (FW: ref) (See further details in Methods). These measurements were made in a representative Nymphalidae species, and used to preliminarily assess the following:

1. the variation in subcostal vein inflation across species and taxa
2. the relationship between the Sc vein inflation and presence or absence of an ear
3. the relationship between vein inflation size and body size (see below)

3.2.4. Do small butterflies tend to have larger inflations?

Chapter 2 posed the hypothesis that pronounced veins might be better developed in smaller butterflies. Smaller insects face difficulty with sound detection and localization due to physical restraints in detecting low frequencies among background interference, especially from farther distances. Their small size creates difficulty with directional hearing, due to the physical constraints in being able to derive sufficient interaural time and intensity differences (ITDs and IIDs, respectively) between both ears (not being able to hear which side the sound is louder, from the sound's source) (Bennet-Clark, 1998; Schmidt & Romer, 2013). In small insects, the distance between ears is smaller than the sound of interest's wavelength such that the animal's body is too small to affect the sound wave significantly as it passes and all or most of the sound diffracts around the body. Thus, the diffraction is not enough to cause difference in pressure cues needed for hearing (Windmill & Jackson, 2016). Further, ears with small surface area simply don't receive as much pressure displacement as larger ears, adding challenges with sound sensitivity and tuning (Windmill & Jackson, 2016). Thus, in butterflies these inflated veins may have evolved as an adaptation for relatively smaller species, and function as hearing aids to overcome the challenge of detecting low frequency sounds. This is seen in many insects such as the *Ormia* fly, who needs to amplify and localize the sounds of its host, the cricket, which is much larger than it. Thus, they have evolved an intertympanal bridge that rocks and bends to allow for sound localization and amplification (Windmill & Jackson, 2016). Adaptations have also been seen in crickets, in which smaller crickets were found to compensate for their physically unfavourable (wavelength to sound) size for hearing by having relatively larger acoustic vesicles (Schmidt & Romer, 2013). In this

chapter, I will test this hypothesis by examining the relationship by wing vein size in butterflies with ears, focusing on Satyrinae.

There are four main goals/objectives:

*Objective 1***:** Test the hypothesis that enlarged veins are associated with hearing organs.

Predictions:

i. Species with inflated veins also have tympanal ears.

ii. Those without ears lack vein inflations.

These predictions will be tested by examining the morphology of Nymphalidae species, with a focus on Satyrinae and (a) scoring the presence or absence of an ear; (b) scoring the size of the forewing veins in two ways: using a forewing: hindwing vein ratio and using a forewing: reference vein ratio.

*Objective 2***:** To examine the taxonomic distribution of vein inflations based on ratio, and the presence/absence of ears.

The following will be informally mapped onto an existing phylogeny:

- Categorical ratios for veins as being large (inflated), small (not), or thick (uncertain)
- Ears (presence/absence/uncertain)

Objective 3: Test the hypothesis that vein inflation size is correlated to body size.

Predictions:

i. Relatively smaller species will have relatively larger vein inflations.

ii. Species with more than one enlarged vein will all be relatively small.

Trends of body size index and vein inflation size will be examined in general and in relation to certain groups, such as tribe, subfamily, and presence/absence of ear. It is acknowledged that this thesis will be assessing these trends only in an exploratory manner; upon further concrete evidence on assessment of a vein inflation, it is recommended to also assess these trends with accounting for phylogeny, using phylogenetic generalized least squares. (e.g. Kang et al. 2017; Thiagavel et al. 2018, see Discussion).

Objective 4: Determine if subcostal vein inflations exhibit sexual dimorphism.

This objective will be performed using selected species for which I had several specimens, *Cercyonis pegala* (male: n=15, female: n=15) (*3.4.4. Results*), and *Coenonympha tullia* (male: n=3, female: n=2) (Appendix 2).

3.3. Methods

3.3.1. Specimens

I collected data from 144 individuals from 79 species belonging to subfamily Satyrinae, and 6 outgroup subfamilies, Biblidinae, Charaxinae, Heliconiinae, Danaiinae,

Nymphalinae, and Apaturinae. Specimens were obtained from (1) the Yack lab at Carleton University, Ottawa, ON, (2) the Kawahara lab at the Florida Museum of Natural History, University of Florida, Gainesville, FL, and (3) live collections by Penghui Sun and Jayne Yack from various places in and around Ottawa, Almonte, and Perth, ON (Table A2.1). Caught species were identified using field guides (Layberry et al. 1998; Opler et al. 1998; Opler et al. 1999) and donated species were identified with the details of the tags on their envelopes (Table A2.1). Both males and females were sampled when possible. Sex was determined using genital morphology.

3.3.2. Morphology of body size, wing vein inflations, and ears

Three categories of morphological features were measured including body size, wing vein inflations, and ear state. The general procedure for taking measurements for each specimen involved first a photograph and measurements of the entire body, then vein measurements, and then ear measurements. Each of these is described in detail in the subsections below. Measurements for a few species were performed as practice to ensure accuracy, by measuring three times to reduce error as follows: Set up specimen under microscope camera, take picture and measurement, remove specimen, readjust, take picture and 2nd measurement, remove specimen and readjust, and take picture and 3rd measurement. The final recorded measurement is the mean of the 3 values taken (e.g. Appendix 2). After confidence from the practice species that all three values for a measurement agreed with each other, I proceeded to complete the rest of the measurements.

3.3.2.1. Body size

To assess body size, the following measurements were taken on both sides of each specimen (except for sides inaccessible or unavailable):

Forewing length: Forewing length was measured as the length from the insertion of the wing on its costal margin to its apex (Figure 3.1A). Photographs of the butterfly against a ruler for scale were taken using a Canon Powershot G16 (Tokyo, Japan), and were measured using ImageJ from Java (v1.51, USA).

Forewing surface area: Forewing surface area was measured as the surface area of the entire forewing, stopping at the insertion of the wing (Figure 3.1A). Photographs were taken using a Canon Powershot G16 (Tokyo, Japan), and were measured using ImageJ from Java (v1.51, USA).

Thorax width: The thorax width was measured at the point of insertion of the forewings as a reference (Figure 3.1B) and was measured using a Fisher Scientific digital calipers (model number 15-077-957, USA).

Forewing length, forewing surface area, and thorax width were used to create a body size index due to their higher robustness and predictive power of true body size (Garcia-Barros, 2015) (See also Analyses Section*: 3.2.4.)*.

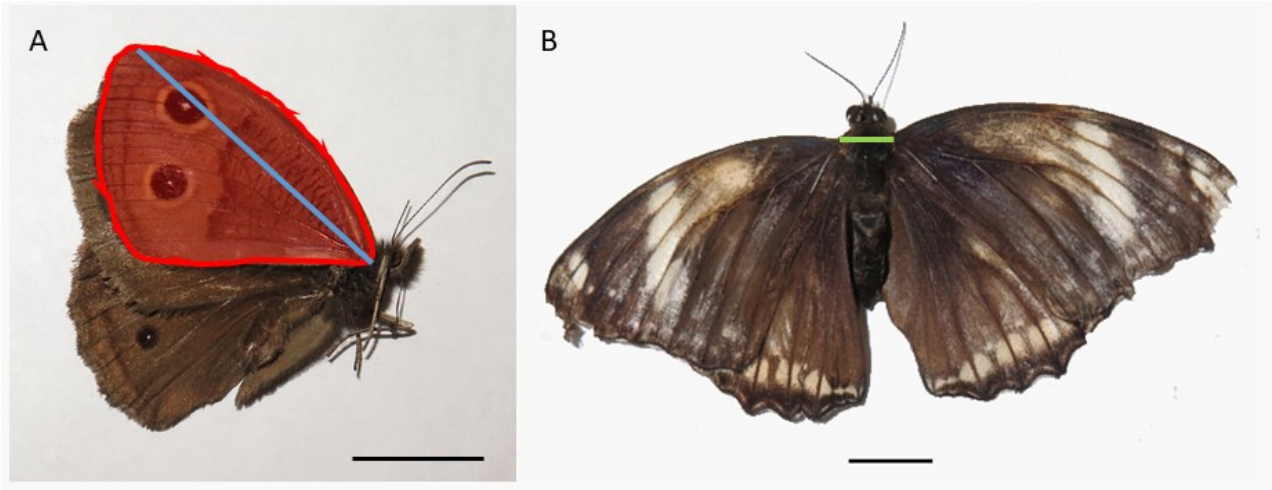


Figure 3.1. Example of where body size measurements were taken. (A) *Cercyonis pegala*, showing forewing length (blue line) and forewing surface area (red area). Scale bar: 10 mm. (B) *Hypolimnas bolina,* showing thorax width (green line). Scale bar: 10 mm.

3.3.2.2. Wing veins

To assess the degree of inflation of the Subcostal wing vein, the following measurements were taken on both sides of each specimen (except for sides inaccessible or unavailable). Photographs were taken using a light microscope (Leica M205C, Germany) equipped with a camera (Leica DMC4500, Germany), and measured using Leica Application Suite (v4.8.0, Germany).

Forewing width: After descaling the forewing if necessary, I photographed the forewing's veins. The widest widths of the subcostal, cubital, and anal veins were measured (Figure 3.2A).

Hindwing width: After descaling the hindwing if necessary, I photographed the hindwing's veins. The widest widths of the subcostal, cubital, and anal veins were measured (Figure 3.2B).

Reference vein width: The distal part of the forewing was photographed and the widest width of radial vein on the forewing was measured just before it splits (intersection of R1 and R2) to represent the reference vein width (figure 3.2C).

These measurements were used to assess the relative size of the Subcostal vein, using two ratio methods to quantify the vein size:

Ratio method 1 – Hindwing vein ratio: The subcostal vein in the forewing is compared to the subcostal vein in the hindwing.

Ratio method 2 – Reference vein ratio: The subcostal vein is compared to the reference vein measurement.

Ratio method 1 follows methodology of a previous student (Hall, 2014). She chose to do a FW:HW ratio "because there is no ear associated with the hindwing" and from her plots and visual observation, she found that all non-eared species did not display vein inflations and were all under the ratio of 3:1. Thus, she concluded that a FW:HW ratio greater than 3 is to be considered inflated whereas a ratio below 3 is to be considered non-inflated. Ratio method 2 is performed to confirm that using the hind wing veins is not confounded with a variation of development differing from the forewing veins, and to compare the vein to another on the forewing. Ideally, I aimed to take a measurement also on the subcostal vein but further down, but was not able to find a consistent place, as the subcostal vein does not intersect with any other veins, and often tapers off into the dorsal part of the wing before even being parallel with another intersection. Thus instead, I used the widest region of the forewing radial vein, which is the next closest vein to the subcostal, just before it splits (intersection of R1 and R2) to represent a reference vein

width. This data was treated as a continuous variable for assessing the relationship between body size and inflations and examining sexual dimorphism in this trait. Ratios were categorized by size for mapping to assess taxonomic distribution and relating to presence/absence of ears (See *3.4.1. Results*)

In addition to Sc vein measurements, two other wing vein features were noted for each specimen:

Internal structure: the forewing veins were assessed to see if they have any structures inside them. These internal structures were classified based on a previous students' categories: Honeycomb, Irregular, Spiral, or None (Stunden, 2014) (See *3.4.1.3. Results*).

Other vein inflations: If other wing veins seemed inflated- If the cubital and/or the anal veins were also noticeably enlarged or swollen prominently, this was also noted.

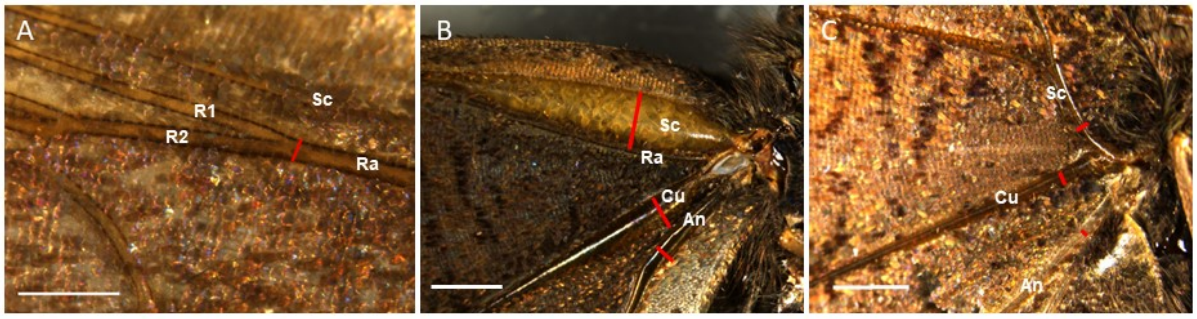


Figure 3.2. Example of how and where wing vein measurements were taken (*Ceryconis pegala*). Red lines are measurements. (A) Reference vein measurement on radial (Ra) vein, taken just before intersection of R1 and R2. Subcostal (Sc) vein (only the posterior part of the vein is shown here), which does not interact with any other veins. Scale bar: 1mm. (B) Forewing vein measurements, for subcostal (Sc), cubital (Cu), and anal (An) veins. Radial (Ra) vein also shown, posterior subcostal vein. Scale bar: 1 mm. (C) Hindwing measurements, for subcostal (Sc), cubital (Cu), and anal (An) veins. Scale bar: 1 mm.

3.3.2.2. Ears

Each specimen was assessed for the presence or absence of a tympanal ear on both left and right sides, contingent on availability. After descaling the forewing if necessary, the base of the forewing was photographed and examined to determine if there

was no ear (Figure 3.3A), an uncertain or intermediate type ear (Figure 3.3B), or a well-developed ear (Figure 3.3C). The state of the tympanal ear was categorized based upon previously established criteria:

Criteria to determine classification of ear (from Preston (2013) and Hall (2014)):

Well-defined or well-developed VO (level 2): A well-defined VO is bordered on three out of four sides by the base of anterior and posterior branches of the cubital vein, as well as the anal vein on the proximate end of the forewing. The cuticular ring is rigid and a symmetrical oval with the long axis traveling from proximal towards distal and the short axis from anterior to posterior. The outer tympanal membrane is ovular as well and clearly bordered by the cuticular ring (Figure 3.3A). It can vary between translucent and opaque.

Uncertain VO (level 1): In this condition, the VO must be bordered on at least two sides by either the anterior and posterior branches of the cubital vein and/or the anal vein. The shape of the cuticular ring manifests as an irregular circle. The membrane itself is asymmetric and the borders of the chitinous ring are not apparent (Figure 3.3B). The membrane can vary between translucent and opaque.

Absent VO (level 0): The VO is completely absent. There is no bifurcation of the cubital vein at its proximate end. In some cases, there is a structure known as the cubital plate at the base of the cubital vein, but no tympanal membrane is present (Figure 3.3C).

This data was then used to determine the relationship between presence/absence of ear and wing vein inflation, as well as to map onto phylogeny.

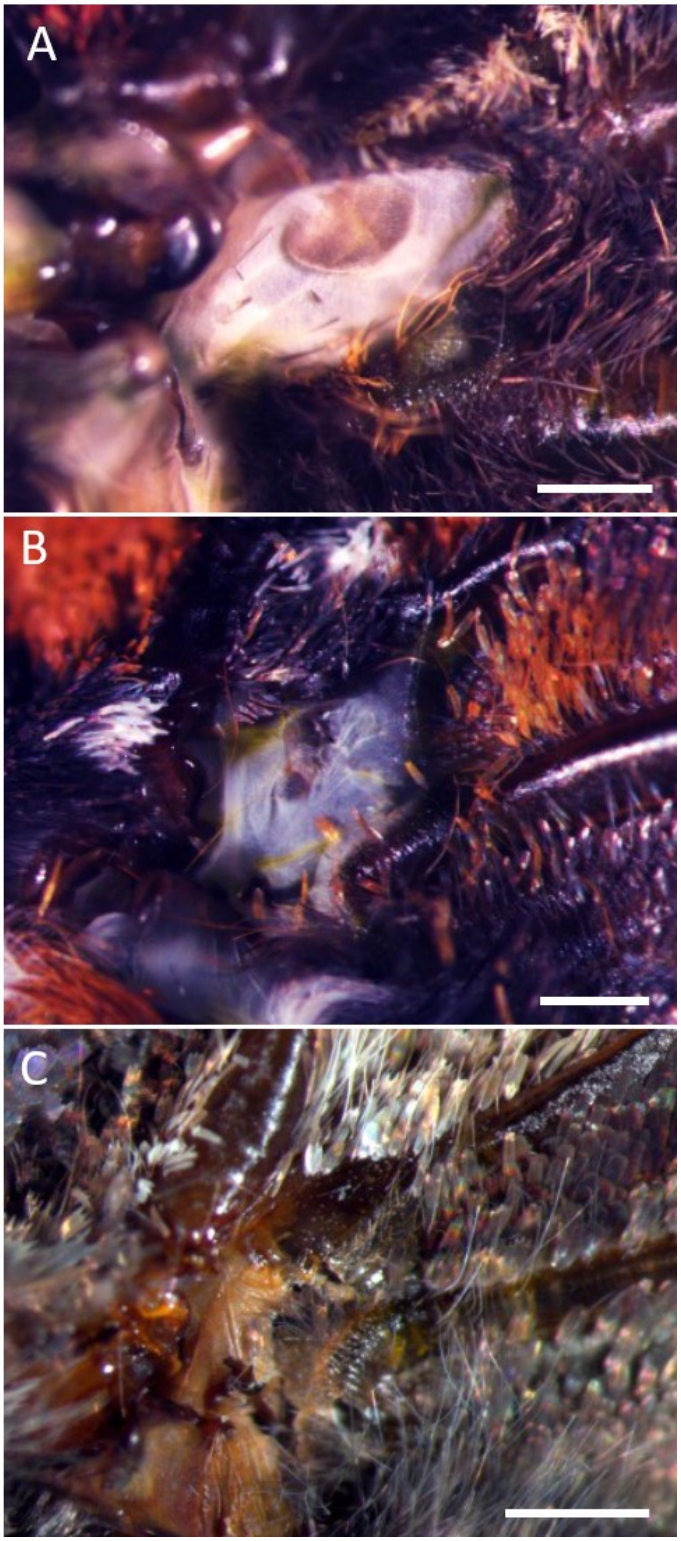


Figure 3.3. Representative species showing the categories of ears. Scales have been removed at the base of the forewing on the ventral side. (A) Absent: *Vanessa rubria*. Scale bar = 500 µm. (B) Uncertain: *Siproeta epaphus*. Scale bar = 500 µm. (C) Well-defined: *Morpho micropthalmus.* Scale bar = 500 µm. (from Hall, 2014).

3.3.3. Taxonomic distribution of subcostal vein and ears

I aimed to examine the taxonomic distribution of vein inflations based on ratio, and the presence/absence of ears, amongst the species sampled (Appendix 2: Table A2.1) Within Satyrinae, species were selected and sampled based on covering representation for all tribes and for variation of size. Species from outgroup subfamilies were selected based on specimen availability and variation in distance of phylogenetic relationships to Satyrinae (Charaxinae is the closest sister group to Satyrinae, and Danaiinae and Heliconiinae are the furthest related outgroups to Satyrinae that I had a multitude of specimens for). Wing vein type and ear type will be informally mapped onto well-resolved and robust trees for Nymphalidae (Wahlberg et al. 2009) and Satyrinae (Pena et al. 2006).

a. Wing vein type: Categories of vein ratio (large, small, thick) will be mapped onto the phylogenies to show how wing vein inflations are distributed amongst tested species.

b. Ear type: Absence and presence (both well-developed and uncertain types) of ears will be mapped onto the same phylogenies to show how ears are distributed amongst species, which species have ears, and how the ears are related to the vein inflations. (See *3.3.2.2. Ears*)

3.3.4. Analyses of vein and body size

Although both left and right-side measurements were taken for all values if available, right-side values were used in data analysis due to more availability of right-side values. Due to their high correlated values, left-side values were used when right-

side values were unavailable (Forewing length R^2=0.97, forewing surface area R^2=0.98, subcostal vein width R^2=0.87, FW:HW ratio R^2=0.82, Reference vein ratio R^2=0.89).

3.2.4.1. Relationship between body size and degree of inflation

The relationship between body size (PC1) and vein size ratio was examined, via both raw plots, and ANCOVAs to assess how taxa levels (tribes and subfamilies) affect vein ratio, accounting for body size as a covariate. A body size index was created by performing a principal component analysis (PCA) on the variables forewing length, forewing surface area, and thorax width, to reduce these body size variables to a few dimensions that represent most of the variation in the data. The PCA was performed in Matlab R2017b (v9.3.0.713579). PC1 from this body size index as a body size indicator was used for assessing the relationship between body size and inflations (see *Analyses*). PC1 was used due to the fact PC1 explained most of the variance in body measures (PC1: 99.99%, PC2: 0.0079%, PC3: 0.0004%).

3.2.4.2. Sexual dimorphism of subcostal veins, ears, and body size

Sexual dimorphism in wing vein inflation widths and ratios was assessed for *C. pegala* (male: n=15, female: n=15) (Appendix 2: *C. tullia*). Sexual dimorphism in body size, represented by PC1, was tested using unpaired t-test with unequal variances. If body size is significantly different, analysis of covariance (ANCOVA) will be used to test for significant differences between sexes in vein sizes and ratios, accounting for body size as a covariate ($p<0.05$) (as per methods: Lane et al. 2008).

3.4. Results

3.4.1. Vein and Ear Morphology

All species sampled in this study have representative plates in Appendix 3, showing the butterfly whole, their forewing veins, and their ear (if they have level 1 or 2 ears).

3.4.1.1. Veins

Within the specimens measured, there was variation in size of subcostal veins. Size of the vein was assessed in 2 ways: FW:HW ratio (ratio method 1) and FW: ref ratio (ratio method 2) (See *Methods 3.3.2* and Table 3.1). Ratio method 1 demonstrated ratios ranging from 1.05-14.77, and ratio method 2 demonstrated ratios ranging from 1.53-13.03 (Figure 3.4 A, B). Even among species with more than one specimen, there was variation in vein size and ratios (See *Results 3.4.4* and Appendix 2). The two ratio methods are strongly correlated ($R^2 = 0.81$) (Figure 3.4C). For purposes of simplicity, this chapter presents the results from ratio method 1 (hindwing vein ratio), and corresponding data on ratio method 2 (reference vein ratio) are presented in Appendix 2.

3.4.1.2. Vein categories

For purposes of mapping on phylogeny and assessing the relationship between the presence of ears and vein inflations (*Results 3.4.3, 3.4.5*), it was necessary to roughly categorize the vein inflations, keeping in mind that there is no functional basis for these categories at present. In reviewing the variation of vein inflations, it became clear that 3/79 species had 'high ratio' veins did not 'appear' to be inflated in the 'classic' sense

('classic' being that they did not have a prominent bulge or elliptical shape) (e.g. Figure 3.7). Thus, further criteria were applied to categorize large, small, or thick veins, besides just the high (>3:1) ratio: the vein having an elliptical shape, and the vein passing assessment by independent observer(s). The elliptical shape criteria are fulfilled when the vein is evidently takes on the shape of an ellipse in some form at its base, instead of being thick and linearly tapers off. To strengthen the shape criteria, the independent observer(s) criteria is fulfilled when independent observer(s) look at the vein and determine if it is a 'classic' inflation to their own perspective.

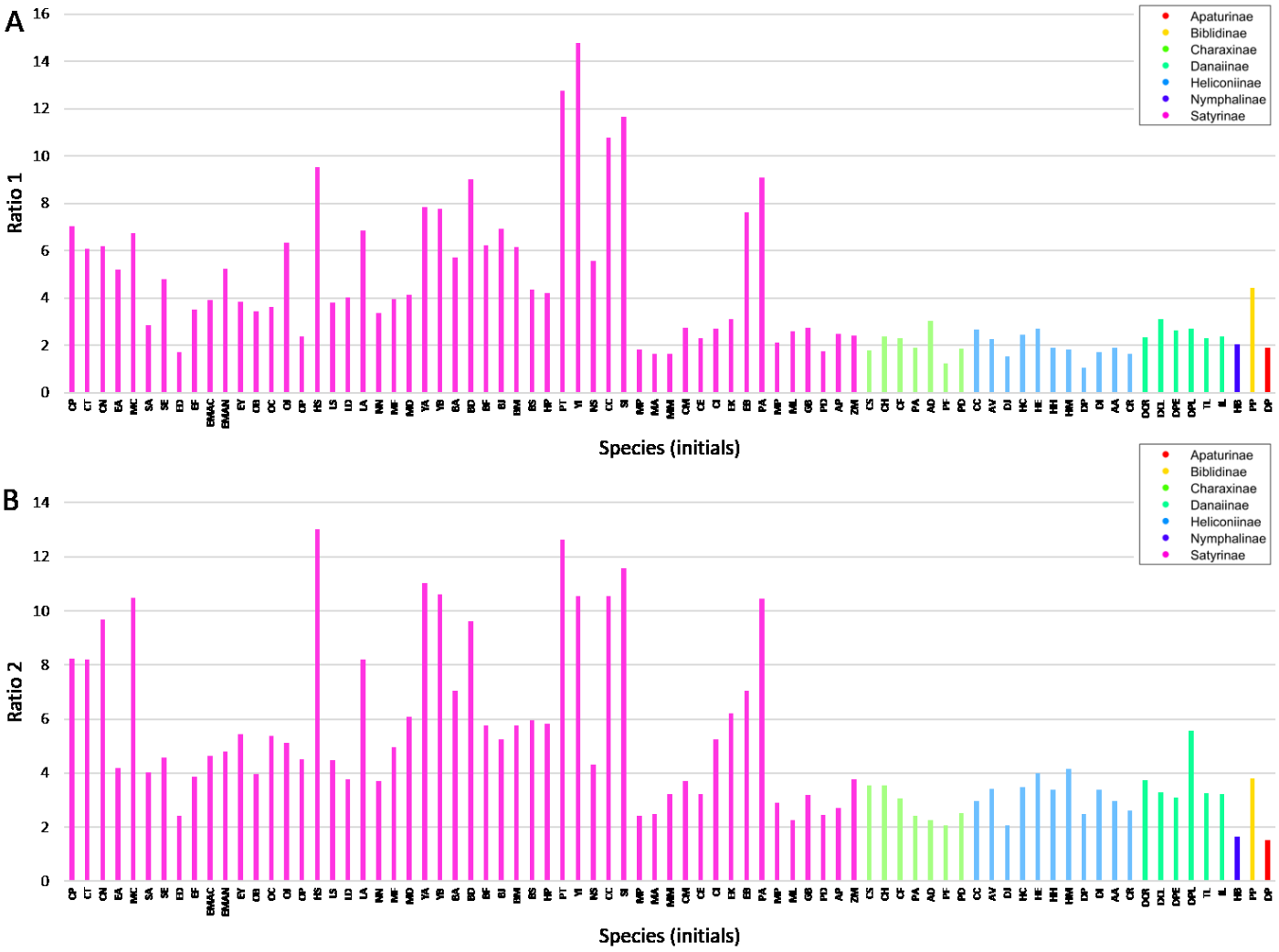
Therefore, for purposes of categorization, the following criteria were applied:
(1) large veins have a ratio close to 3:1, are elliptical shaped, and pass assessment by independent observer(s) (Figure 3.5)
(2) small, which are obviously not inflated (Figure 3.6), and pass assessment by independent observer(s)
(3) thick, which have a high ratio >3, but are not obviously inflated, and pass assessment by independent observer(s) (Figure 3.7).

In total, 39 species were categorized as "large." Three species were categorized as thick, *Panacea prola, Archaeprepon demophon,* and *Danaus cleophile* (Figure 3.7). The remaining 37 species were categorized as small (non-inflated) (Figure 3.6). Note that these categories were made only for purposes of mapping, and to provide preliminary insight into size and inflation variation, and relationship with ears. It will be necessary in the future to further quantitatively qualify the vein with other criteria, such as geometric morphometrics, and to map the trait on accounting for phylogenetic biases (see Discussion *3.5.1, 3.5.3*).

Table 3.1. Summary of species' vein sizes and ear states. † L: Large, S: Small, T: Thick

Subfamily	Tribe	Species	Sc Vein (Ratio 1)	Sc Vein (Ratio 2)	Sc Vein Category (L, S, T) †	Ear State (0/1/2)	Internal structures	Other inflated veins?
Satyrinae	Satyrini	*Cercyonis pegala*	7.04	8.22	L	2	Honeycomb	N
Satyrinae	Satyrini	*Coenonympha tullia*	6.09	8.21	L	2	Spiral	Y: Cu, An
Satyrinae	Satyrini	*Coenonympha nipisquit*	6.20	9.67	L	2	Spiral	Y: Cu, An
Satyrinae	Satyrini	*Enodia anthedon*	5.22	4.18	L	2	None	N
Satyrinae	Satyrini	*Megisto cymela*	6.76	10.48	L	2	Irregular	N
Satyrinae	Satyrini	*Satyrodes appalachia*	2.88	4.04	L	2	None	N
Satyrinae	Satyrini	*Satyrodes eurydice*	4.81	4.58	L	2	None	N
Satyrinae	Satyrini	*Erebia discoidalis*	1.73	2.42	S	2	None	N
Satyrinae	Satyrini	*Erebia fasciata*	3.51	3.87	L	2	None	N
Satyrinae	Satyrini	*Erebia mackinleyensis*	3.93	4.63	L	2	None	N
Satyrinae	Satyrini	*Erebia mancinus*	5.25	4.81	L	2	None	N
Satyrinae	Satyrini	*Erebia youngi*	3.85	5.44	L	2	None	N
Satyrinae	Satyrini	*Oeneis bore*	3.44	3.97	L	2	None	N
Satyrinae	Satyrini	*Oeneis chryxus*	3.64	5.37	L	2	Irregular: Other	N
Satyrinae	Satyrini	*Oeneis jutta*	6.34	5.13	L	2	Irregular: Other	N
Satyrinae	Satyrini	*Oeneis polixenes*	2.40	4.51	L	2	None	N
Satyrinae	Satyrini	*Hermeuptychia sosybius*	9.54	13.03	L	2	Irregular	Y: Cu
Satyrinae	Satyrini	*Lethe diana*	4.05	3.78	L	2	None	N
Satyrinae	Satyrini	*Lethe sicelis*	3.82	4.48	L	2	None	N
Satyrinae	Satyrini	*Lopinga achine*	6.87	8.21	L	2	Honeycomb	N
Satyrinae	Satyrini	*Neope niphonica*	3.38	3.70	L	2	None	N
Satyrinae	Satyrini	*Mycalesis francisca*	3.97	4.96	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Minois dryas*	4.16	6.09	L	2	Irregular: Other	N
Satyrinae	Satyrini	*Ypthima albida*	7.86	11.02	L	2	Irregular	Y: Cu
Satyrinae	Satyrini	*Ypthima baldus*	7.77	10.60	L	2	Irregular	N
Satyrinae	Satyrini	*Bicyclus aurivilli*	5.72	7.05	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Bicyclus denina*	9.00	9.62	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Bicyclus funebris*	6.22	5.76	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Bicyclus jeffreyi*	6.92	5.26	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Bicyclus mandanes*	6.17	5.75	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Bicyclus sebetus*	4.37	5.94	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Heteropsis perspicua*	4.23	5.83	L	2	None	Y: Cu, An
Satyrinae	Satyrini	*Pseudonympha trimenii*	12.78	12.62	L	2	None	N
Satyrinae	Satyrini	*Ypthimomorpha itonia*	14.77	10.55	L	2	Honeycomb	N
Satyrinae	Satyrini	*Ninguta schrenkii*	5.58	4.32	L	2	None	N
Satyrinae	Satyrini	*Cassionympha cassius*	10.79	10.56	L	2	Honeycomb	N
Satyrinae	Satyrini	*Stygionympha irrorata*	11.67	11.56	L	2	Honeycomb	N
Satyrinae	Morphini	*Morpho amathonte*	1.63	2.49	S	2	None	N
Satyrinae	Morphini	*Morpho microthalmus*	1.66	3.24	S	2	None	N

Satyrinae	Morphini	*Morpho peleides*	1.84	2.41	S	2	None	N
Satyrinae	Brassolini	*Caligo eurilochus*	2.31	3.23	S	2	None	N
Satyrinae	Brassolini	*Caligo illioneus*	2.71	5.23	S	2	None	N
Satyrinae	Brassolini	*Caligo memnon*	2.75	3.70	S	2	None	N
Satyrinae	Elymiini	*Elymniosis bammakoo*	7.64	7.04	L	2	None	N
Satyrinae	Elymiini	*Elymnias kanekei*	3.10	6.20	L	2	Irregular: Other	N
Satyrinae	Haeterini	*Pierella astyoche*	9.11	10.44	L	2	Spiral	N
Satyrinae	Melanitini	*Melanitis leda*	2.60	2.25	S	2	None	N
Satyrinae	Melanitini	*Melanitis phedima*	2.14	2.90	S	2	None	N
Satyrinae	Melanitini	*Gnophodes betsimena*	2.75	3.19	S	2	None	N
Satyrinae	Dirini	*Paralethe dendrophilus*	1.76	2.47	S	0	None	N
Satyrinae	Amathusiini	*Amathusia phidippus*	2.48	2.70	S	1	None	N
Satyrinae	Zetherini	*Zethera musa*	2.44	3.78	S	0	None	N
Charaxinae	Charaxini	*Charaxes howarthi*	2.38	3.56	S	2	None	N
Charaxinae	Charaxini	*Charaxes subornatus*	1.80	3.53	S	1	None	N
Charaxinae	Charaxini	*Consul fabius*	2.29	3.07	S	2	None	N
Charaxinae	Charaxini	*Polyura athamas*	1.90	2.42	S	2	None	N
Charaxinae	Charaxini	*Archaeprepon demophon*	3.03	2.26	T	1	None	N
Charaxinae	Prothonini	*Prothoe franck*	1.26	2.06	S	1	None	N
Charaxinae	Pallini	*Palla decius*	1.87	2.51	S	1	None	N
Heliconiinae	Acraeini	*Acraea viola*	2.26	3.41	S	0	None	N
Heliconiinae	Acraeini	*Cethosia cyane*	2.69	2.96	S	0	None	N
Heliconiinae	Heliconiini	*Dione juno*	1.52	2.06	S	0	None	N
Heliconiinae	Heliconiini	*Heliconius charitonia*	2.45	3.48	S	0	None	N
Heliconiinae	Heliconiini	*Heliconius erato*	2.72	3.99	S	0	None	N
Heliconiinae	Heliconiini	*Heliconius hecale*	1.91	3.39	S	0	None	N
Heliconiinae	Heliconiini	*Heliconius melpomene*	1.83	4.16	S	0	None	N
Heliconiinae	Heliconiini	*Dryadula phaetusa*	1.06	2.50	S	0	None	N
Heliconiinae	Heliconiini	*Dryas iullia*	1.70	3.40	S	0	None	N
Heliconiinae	Argyniini	*Argynnis aphrodite*	1.92	2.97	S	0	None	N
Heliconiinae	Vagrantini	*Cirrochroa regina*	1.66	2.61	S	0	None	N
Danaiinae	Danaini	*Danaus chrysippus*	2.33	3.73	S	0	None	N
Danaiinae	Danaini	*Danaus cleophile*	3.13	3.30	T	0	None	N
Danaiinae	Danaini	*Danaus petilia*	2.65	3.09	S	0	None	N
Danaiinae	Danaini	*Danaus plexippus*	2.70	5.56	S	0	None	N
Danaiinae	Danaini	*Tirumala limniace*	2.29	3.26	S	0	None	N
Danaiinae	Danaini	*Idea leuconoe*	2.38	3.23	S	0	None	N
Nymphalinae	Junonini	*Hypolimnas bolima*	2.05	1.66	S	1	None	N
Biblidinae	Ageroniini	*Panacea prola*	4.44	3.80	T	2	None	N
Apaturinae	NA	*Doxocopa pavon*	1.92	1.54	S	2	None	N


A
Ratio 1
Species (initials)
B
Ratio 2
Species (initials)
Apaturinae
Biblidinae
Charaxinae
Danaiinae
Heliconiinae
Nymphalinae
Satyrinae

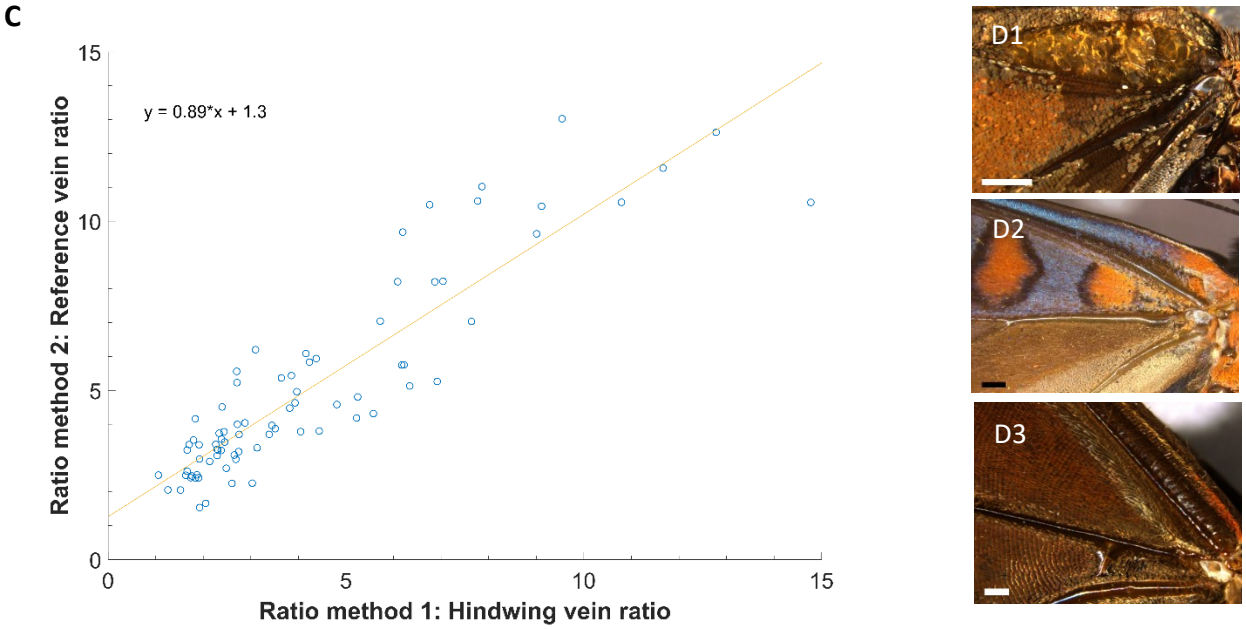


Figure 3.4. Relationship agreement between the two ratio methods used. (A) Vein ratio size for all species using ratio method 1, species ID'd using initials, (B) Vein ratio size for all species using ratio method 2, species ID'd using initials (e.g. *Genus species*: GS), (C) Correlation between two ratio methods, $R^2 = 0.81$, (D1) Example of species with large ratios for either method (*Stygionympha irrorata*, Satyrinae, scale bar = 1 mm), (D2) Example of species with medium ratios for either method (*Panacea prola*, Biblidinae, scale bar = 1 mm), (D3) Example of species with small ratios for either method (*Morpho micropthalmus,* Satyrinae, scale bar = 1 mm).

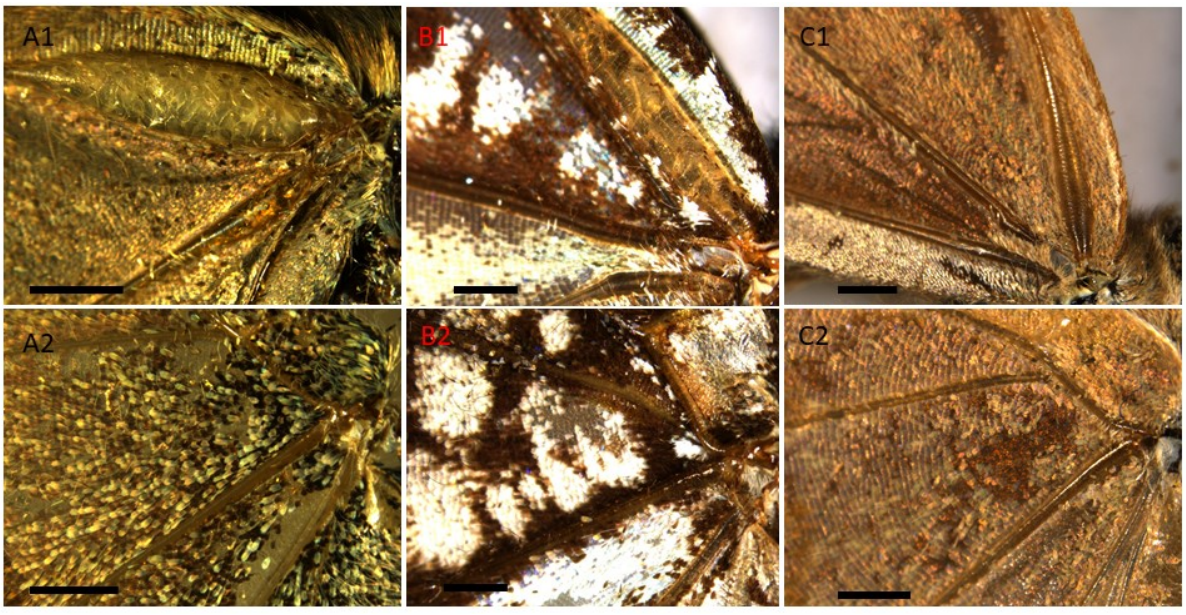


Figure 3.5. Representative specimens showing "large/inflated" type veins. Scales have been removed at the base of the forewing on the ventral side. Top: Forewings; Bottom: Hindwings. All scale bars = 1 mm. (A) *Megisto cymela* (Satyrinae: Satyrini). (B) *Elymnias kanekei* (Satyrinae: Elymiini). (C) *Satyrodes eurydice* (Satyrinae: Satyrini).

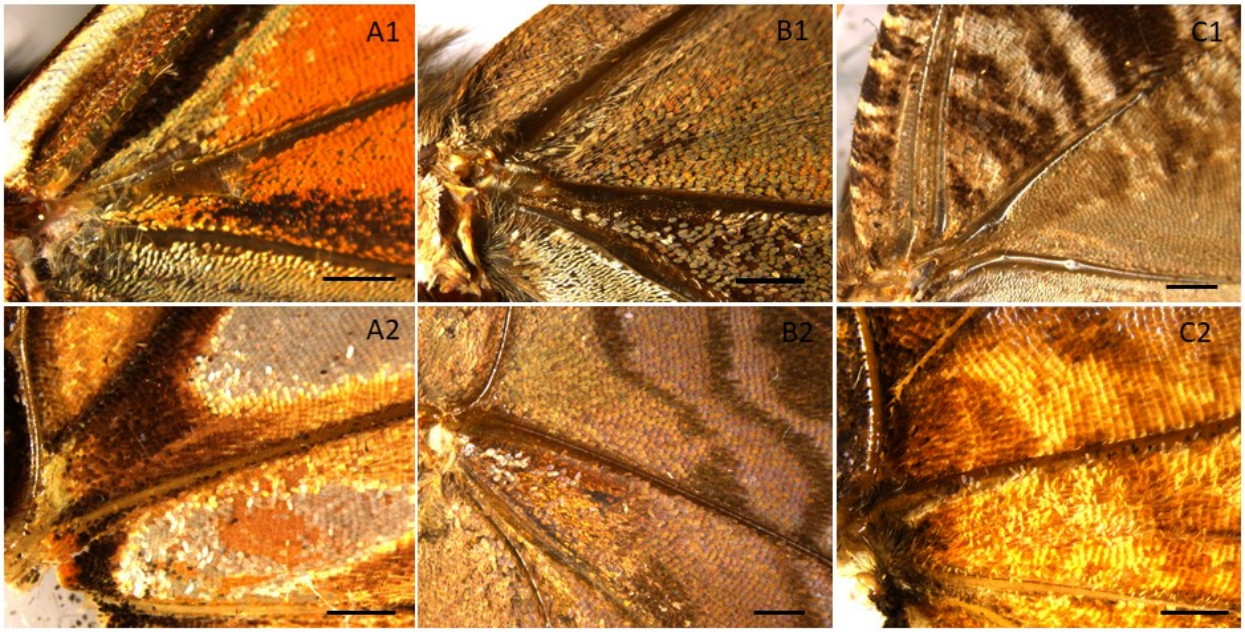


Figure 3.6. Representative specimens showing "small/not inflated" type veins. Scales have been removed at the base of the forewing on the ventral side. Top: Forewings; Bottom: Hindwings. All scale bars = 1 mm. (A) *Dione juno* (Heliconiinae: Heliconiini). (B) *Paralethe dendrophilus* (Satyrinae: Dirini). (C) *Consul fabius* (Charaxinae: Charaxini).

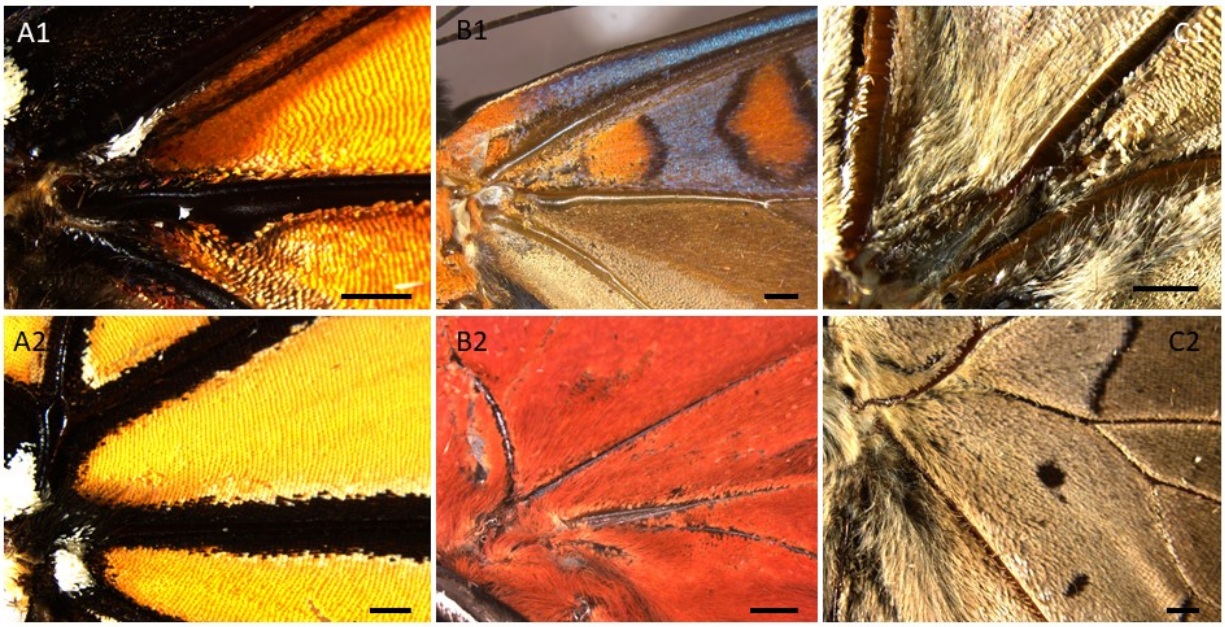


Figure 3.7. Representative specimens showing "uncertain/thick" type veins. Scales have been removed at the base of the forewing on the ventral side. Top: Forewings; Bottom: Hindwings. All scale bars = 1 mm. (A) *Danaus cleophile* (Danaiinae: Danaiini). (B) *Panacea prola* (Biblidinae: Ageroniini). (C) *Archaeprepon demophon* (Charaxinae: Charaxini).

3.4.1.3. Other vein features

Some species exhibited enlarged cubital and anal veins (Figure 3.8, Table 3.1). Within this study, all species that had enlarged cubital and anal veins were in subfamily Satyrinae and tribe Satyrini. Cubital veins were never inflated without the subcostal vein also being inflated, and anal veins were never inflated without the subcostal and cubital veins also being inflated. These veins were only designated as large if they were prominently large and elliptical-shaped, but for the purposes of this study, these veins were not formally quantified or analyzed.

Further, some species were observed to have internal structures within their subcostal wing veins (Table 3.1). Any species with internal structures all had large inflated type wing veins. These are a network of interconnecting layers of tissue within the vein that form a geometric configuration (Figure 3.9) and are fragile and collapse when opened (personal observation). These structures were informally observed and categorized using previously designated categories: Spiral, honeycomb, or irregular (Stunden, 2014). Spiral patterns were characterized by the presence of a spiral staircase pattern (Figure 3.9A). Honeycomb patterns were characterized by a uniform pattern of interconnections (Figure 3.9B). Irregular patterns were characterized by the presence of an irregular pattern (Figure 3.9C). There was wide variation amongst the "irregular" pattern, such as a network of interlocking membranes (Figure 3.9D), and a network similar to "spiral" but with intertwined horse-shoe shaped parts (Figure 3.9E). I also designate species with inflated veins that did not have internal structures as "none" (Figure 3.9F). No species that had thick or small veins had internal structures. One species, *Ypthima albida* also had spiral internal structures within its cubital and anal vein

(Figure A3.35). Having internal structures in cubital or anal veins was not seen in any other species sampled. Among species sampled in this study, all species with internal veins occurred in the subfamily Satyrinae and the tribe Satyrini. Although most specimens had clear intact internal structures if they had them, some older specimens may have had fragile tissues collapse or warp over time, and thus the age of the specimen should be taken into consideration in future studies of these internal structures. Furthermore, as seen with the variation in irregular type internal structures, there needs to be more sampling to determine definitive categories for these internal structures.

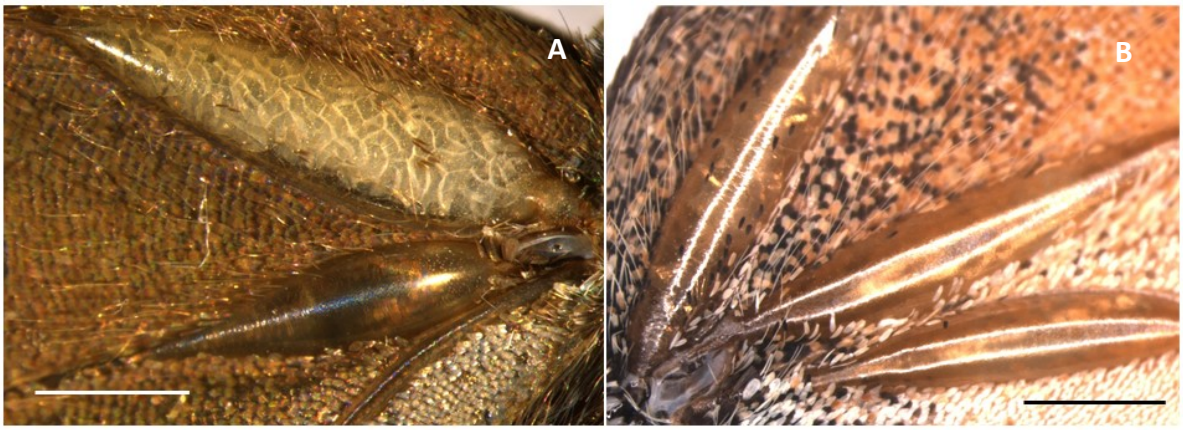


Figure 3.8. Representative specimens showing examples of species with more than one inflated vein. All scale bars = 1 mm. (A) Spiral: *Hermeuptychia sosybius* (Satyrinae: Satyrini). (B) Honeycomb: *Coenonympha tullia* (Satyrinae: Satyrini)

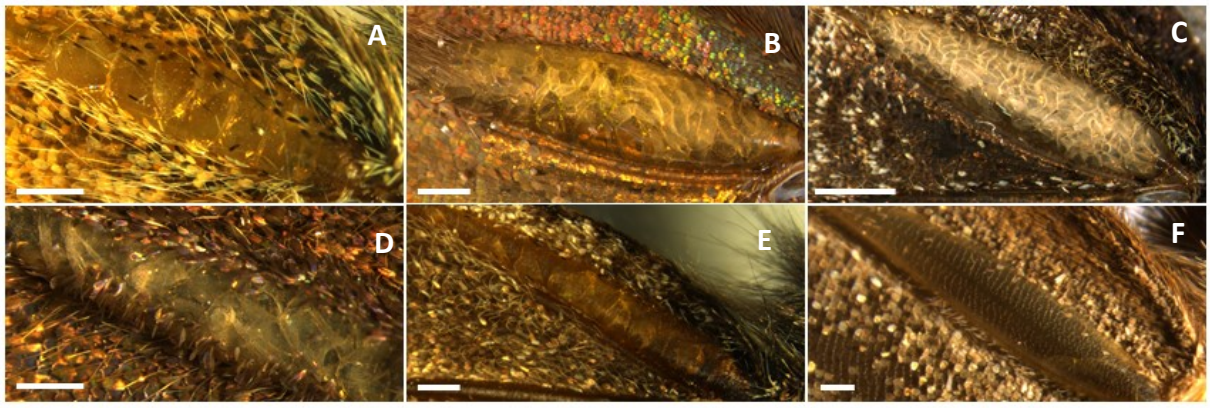


Figure 3.9. Representative specimens showing the categories of internal structures within subcostal veins on the ventral side of the forewing. Scales have been removed at the base of the forewing on the ventral side. All scale bars = 500 µm. (A) Spiral: *Coenoympha nipisquit* (Satyrinae: Satyrini). (B) Honeycomb: *Cassionympha cassius* (Satyrinae: Satyrini). (C) Irregular: *Ypthima baldus* (Satyrinae: Satyrini). (D) Alternative irregular: *Oeneis jutta* (Satyrinae: Satyrini). (E) Alternative irregular: *Minois dryas*. (F) None: *Bicyclus sebetus* (Satyrinae: Satyrini)

3.4.2. Ears

Ear states were categorized based on the 3 previous categories outlined in *Methods,* but even within the same ear state category, there was variation (Figure 3.10). Fifty-five out of all seventy-nine species (69.6%) had well-developed ears, 6 (7.6%) had uncertain ears, and 18 (22.8%) had no ears. The taxonomic distribution of ears will be discussed in *3.4.5.*

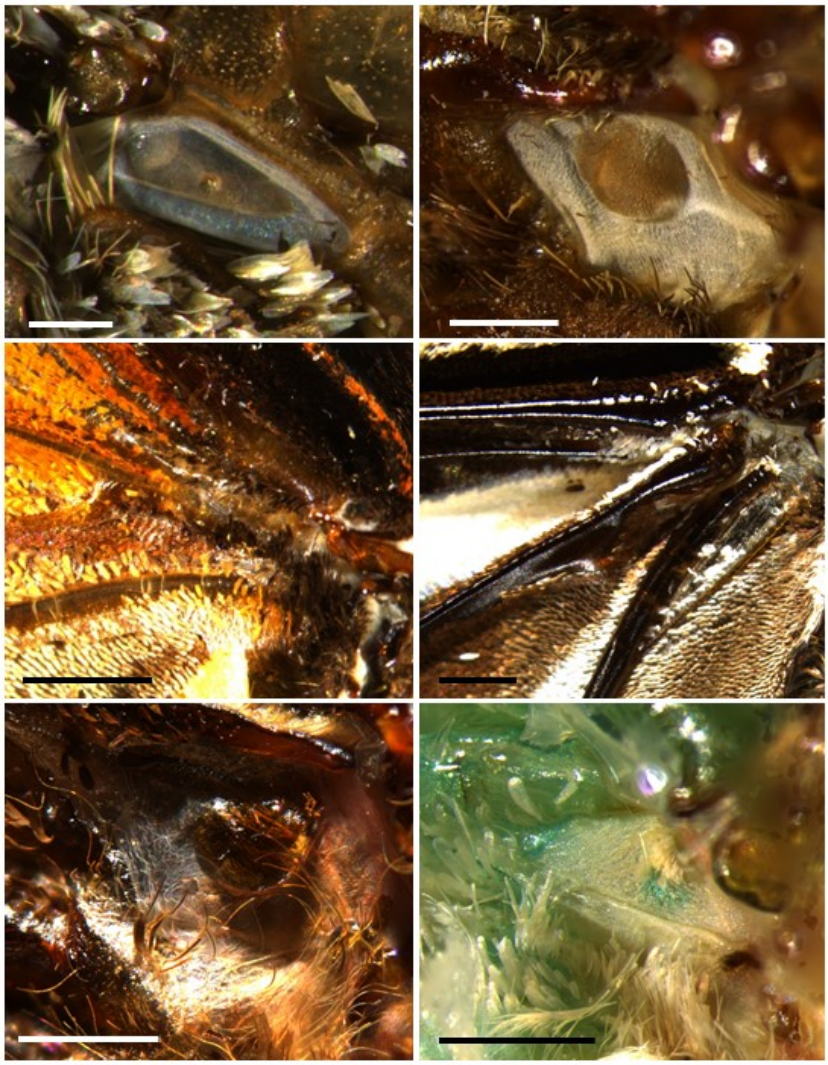


Figure 3.10. Representative specimens showing the categories of ears. Scales have been removed at the base of the forewing on the ventral side. (A) Well-defined: *Ypthima baldus* (Satyrinae: Satyrini). Scale bar = 200 µm. (B) Alternative well-defined: *Morpho microthalmus* (Satyrinae: Morphini). Scale bar = 500 µm. (C) Absent: *Heliconius hecale* (Heliconiinae: Heliconiini). Scale bar = 1 mm. (D) Alternative absent: *Tirumala limniace* (Danaiinae: Danaiini). Scale bar = 1 mm. (E) Uncertain: *Palla decius* (Charaxinae: Pallini). Scale bar = 500 µm. (F) Alternative uncertain: *Charaxes subornatus* (Charaxinae: Charaxini). Scale bar = 500 µm.

3.4.3. Relationship between veins and ears

Among species sampled in this study, all species classified with large veins also have well-developed ears. However, species with ears varied in vein types, including large, small, and thick (Table 3.1, Figure 3.11, 3.12).

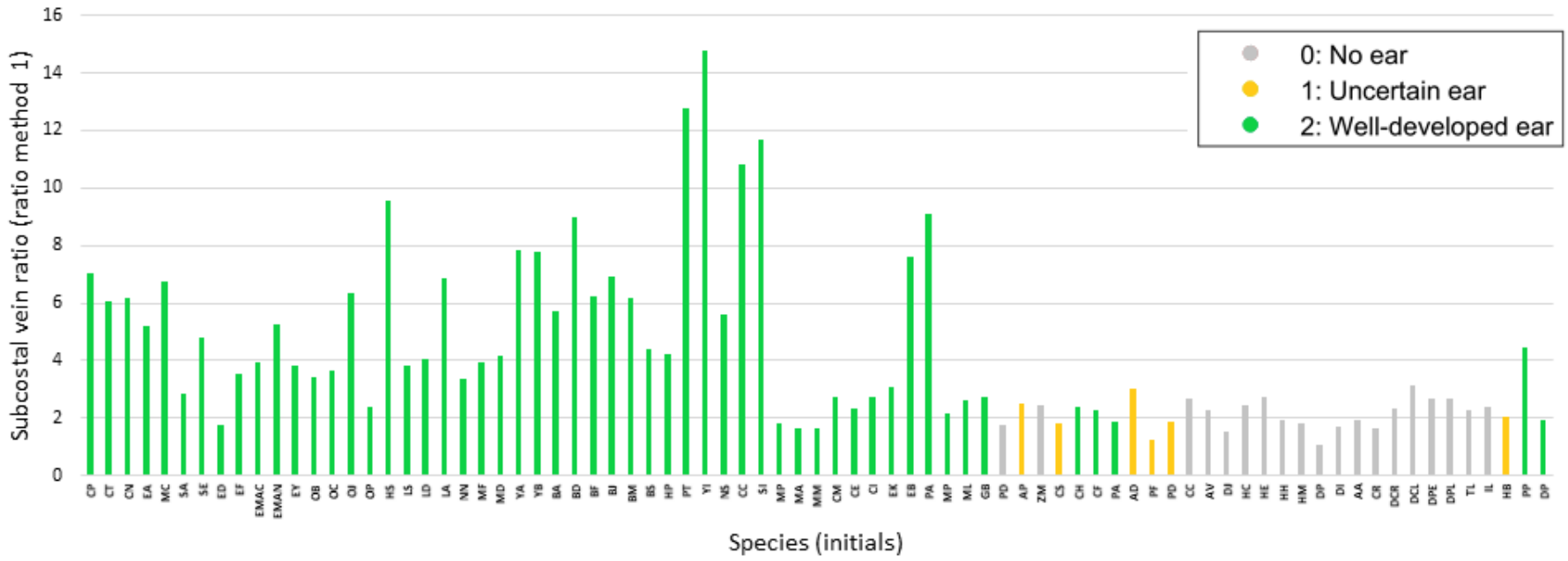


Figure 3.11. Vein ratios types categorized by ear types, using ratio method 1 for all species sampled. Species ID'd by initials (e.g. *Genus species*: GS from Table 3.1).

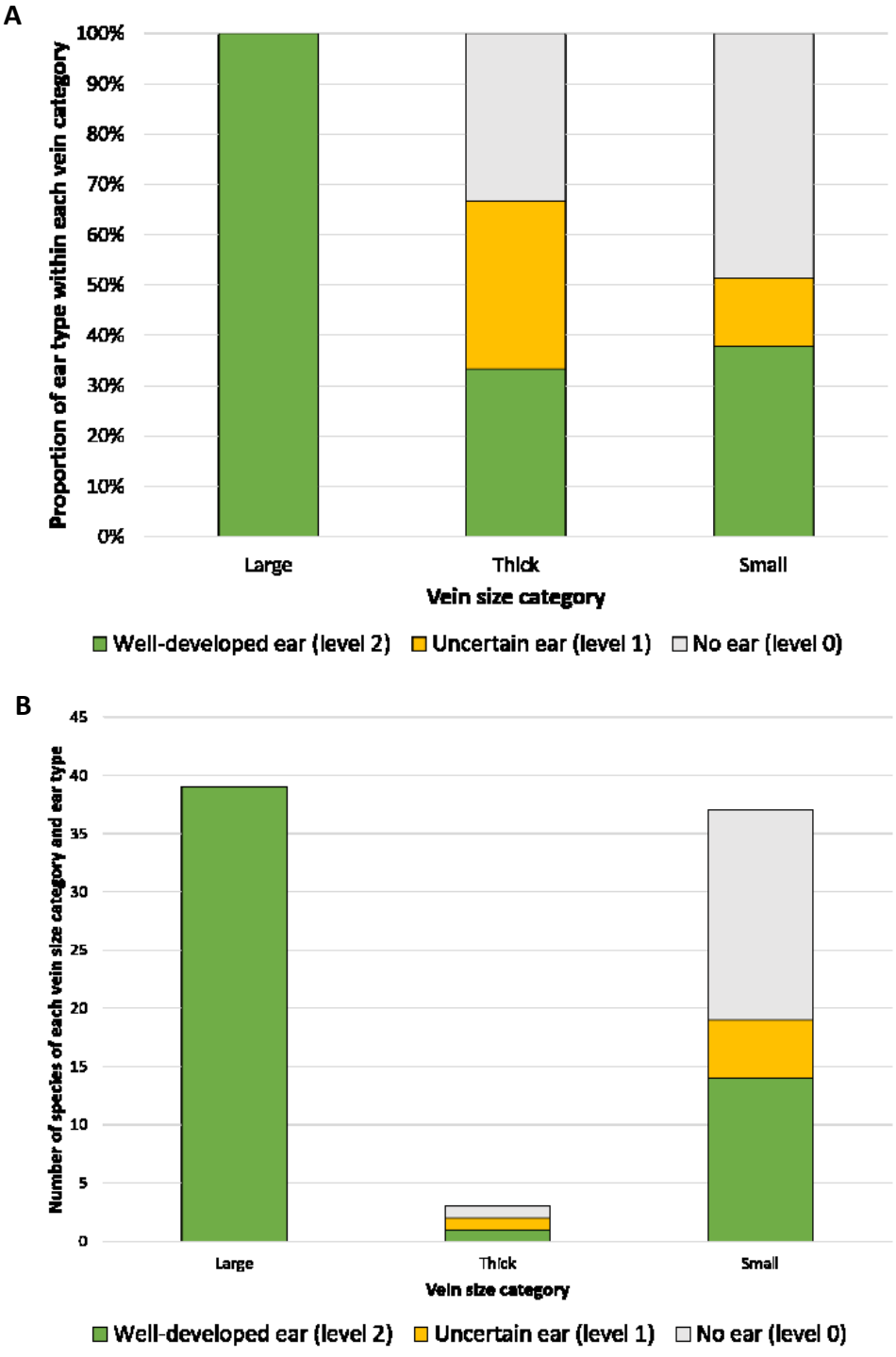
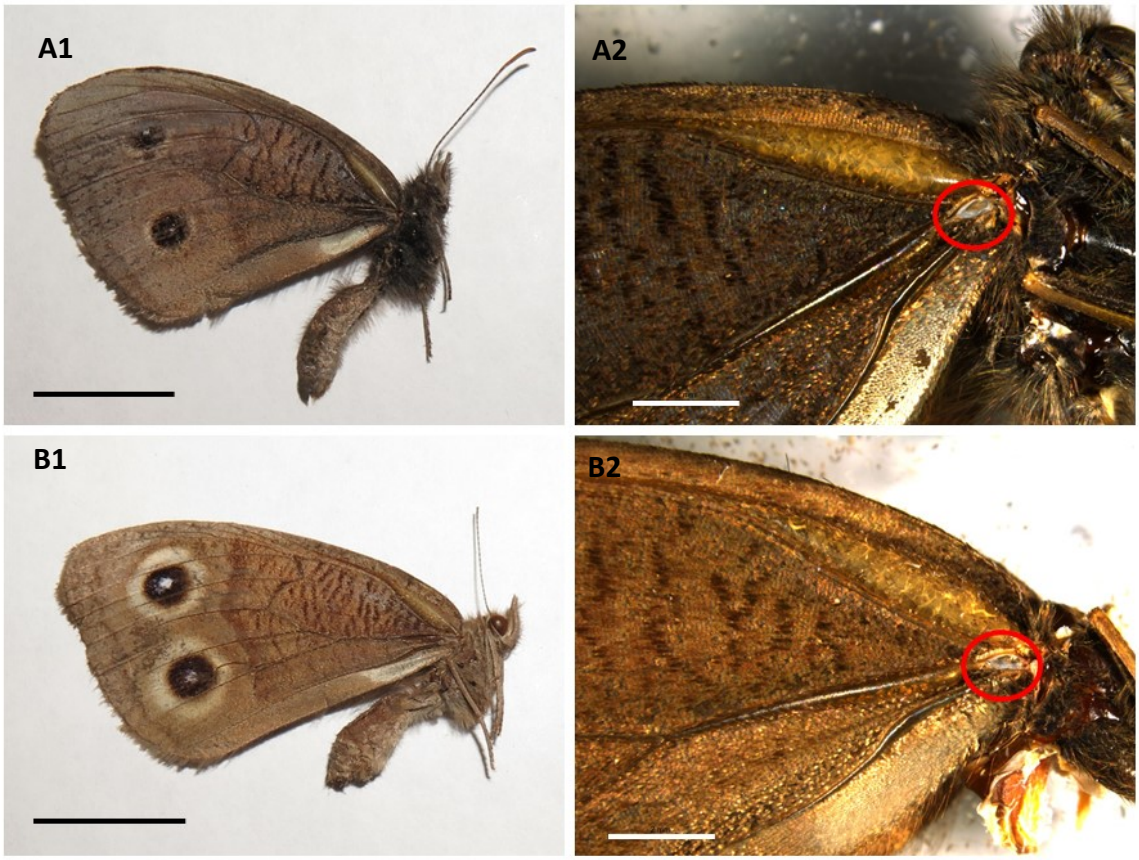


Figure 3.12. Ear types and vein sizes of the species sampled using ratio method 1. (A) Proportion of vein sizes and ear types for all species sampled. (B) Number of species for each vein size and ear type.

3.4.4. Sexual Dimorphism

Sexual dimorphism was tested for two representative species, *Cercyonis pegala* (male: n=15, female: n=15) and *Coenonympha tullia* (male: n=3, female: n=2) (Appendix 2). Body size of *C. pegala* did not exhibit significant difference between males and females, thus t-tests with unequal variances were performed to test sexual dimorphism in vein width and ratio. Vein width exhibited sexual dimorphism, but vein ratio did not (Table 3.2). Male vein width ranged from 974.05-1202.45 µm, with FW:HW ratios ranging from 5.52-8.54. Female vein width ranged from 1053.46-1287.42 µm, with ratios ranging from 4.73-9.34.



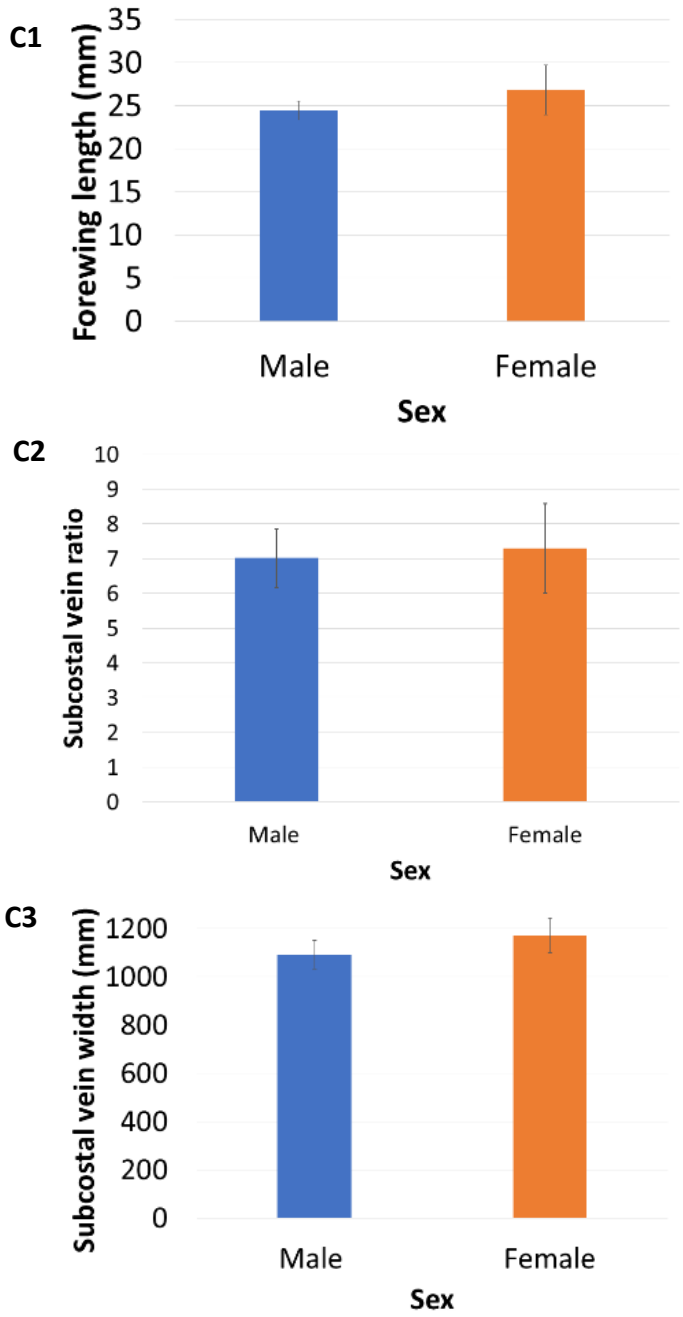


Figure 3.13. Male and female examples of morphology in *C. pegala*. (A1) Whole male on ventral side, hindwing detached, scale bar = 1 mm, (A2) Male forewing, circle denotes ear, scale bar = 2 mm, (B1) Whole female on ventral side, hindwing detached, scale bar = 1 mm, (B2) Female forewing, circle denotes ear, scale bar= 2 mm, (C1) Forewing length between sexes, with standard deviation bars, (C2) Subcostal vein ratio between sexes, with standard deviation bars, (C3) Subcostal vein width, between sexes, with standard deviation bars.

Table 3.2. Sexual dimorphism in body size using PC1 was tested using unpaired t-test with unequal variances, $p<0.05$ taken as significant.[1] Sexual dimorphism in *C. pegala* for vein ratio 1, tested using unpaired t-test, with unequal variances, $p<0.05$ taken as significant.[2]

Subcostal vein width (μm)			**Subcostal vein ratio (ratio method 1)**			**Body size: PC1**		
Male	Female	p-value[2]	Male	Female	p-value[2]	Male	Female	p-value[1]
1091.65 ± 59.08	1173.36 ± 71.64	**0.003**	7.02 ± 0.85	7.30 ± 1.29	0.51	6.96E-14 ± 23.01	-4.69E-14 ± 39.24	1

3.4.5. Taxonomic distribution

Table 3.1 and Figure 3.14 summarize the distribution of occurrence of vein size categories and ear states for ratio method 1. There is variation in the degree of wing vein inflation between species and within tribes. Veins classified as large are mostly found in tribe Satyrini, but also occur in Haeterini and Elymiini. Of the three species classified as "thick", each belonged to a different subfamily. Veins classified as small occurred throughout subfamilies sampled (Table 3.1, Figure 3.14).

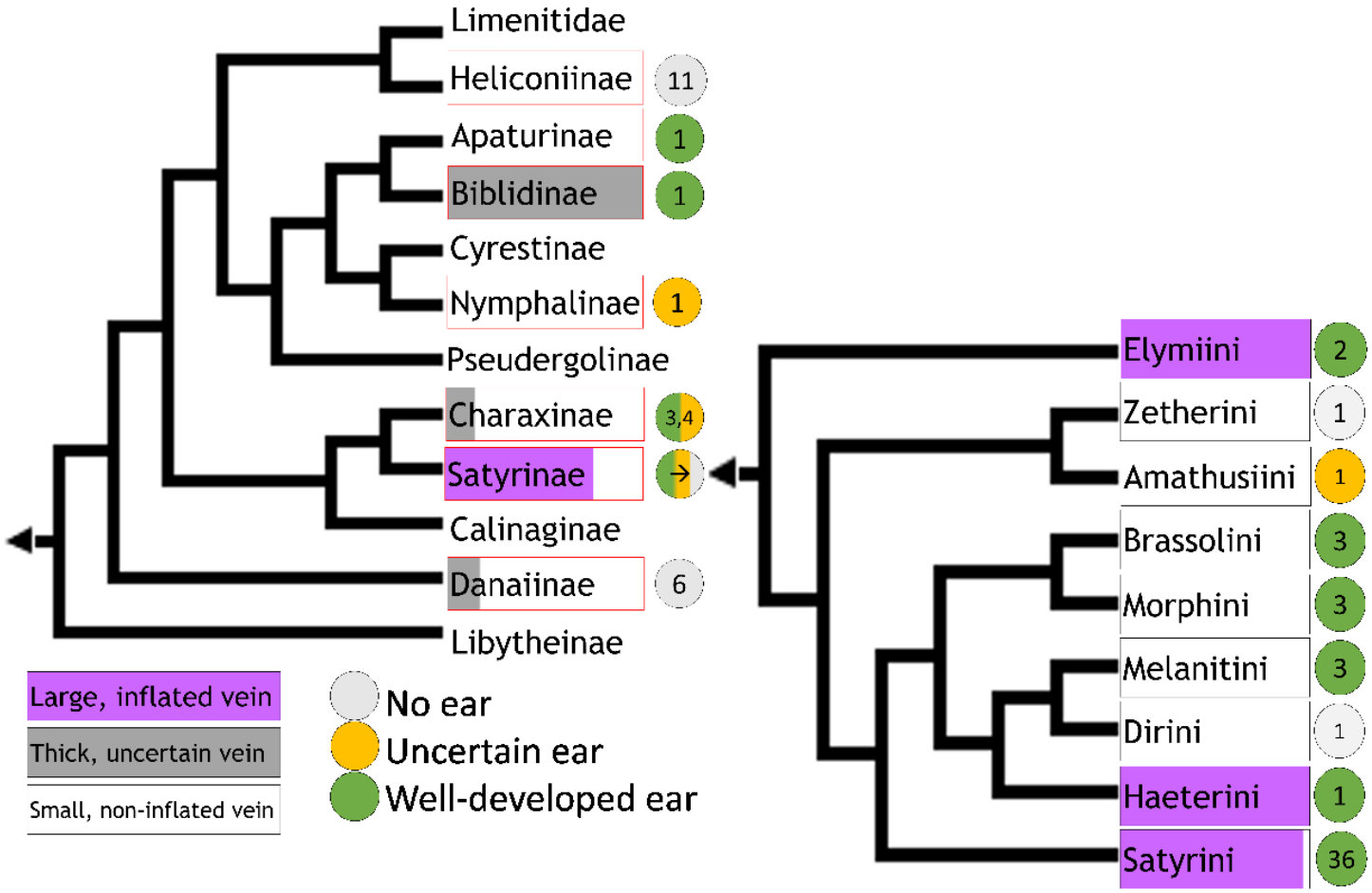


Figure 3.14. Phylogeny of Nymphalidae, with branch of Satyrinae, showing occurrence of vein categories and ear state in each group. Subfamilies sampled in group boxes outlined in red, colours within each group box shows if vein type occurs in that group (proportional to total in group), and colours within circles show if ear type occurs in that group. Numbers inside circles denote number of species sampled in that group, and in coloured parts, of that ear state. All tribes in Satyrinae were sampled. Hall (2014) notes that large inflated veins also occur in Biblidini and Ageroniini tribes for Biblidinae. Trees modified from Pena et al. (2006) and Wahlberg et al. (2009).

3.4.6. Relationship of subcostal vein size to body size

I tested the hypothesis that vein inflations are more pronounced in butterflies with smaller body size. A body size index was created by performing a principal component analysis (PCA) on the variables to reduce these body size variables to a few dimensions that represent most of the variation in the data (Figure 3.15). PC1 explained most of the variance in body measures (PC1: 99.99%, PC2: 0.0079%, PC3: 0.0004%). I used a Nymphalidae phylogeny (Wahlberg et al. 2009) and a Satyrinae phylogeny (Pena et al. 2006) to map the inflated vein trait. For now, I am only presenting plots of raw data without accounting for phylogeny. However, these data allow us to inspect the data to assess relationships between taxonomic group, body size, and presence and absence of ears. ANCOVAs have also been performed to assess how taxa levels (tribes and subfamilies) affect vein ratio, accounting for body size as a covariate. Future work should take this further and incorporate these insights when eventually performing phylogenetic comparative tests to account for phylogenetic bias in phylogenetic relationships between species (See *Discussion 3.5.3*).

Analyses were performed between body size and vein ratio using all species (Figure 3.16 A, B), only within Satyrinae (Figure 3.16 C), and only within Satyrini (Figure 3.16D). In all analyses smaller body size correlated with larger sized veins, and all analyses demonstrated a best relationship fit using a two-term exponential model (Linear: All R^2=0.4682, Satyrinae R^2=0.4077, Satyrini R^2=0.29, Log: All R^2=04519, Satyrinae R^2=0.4546, Satyrini R^2=0.2453), over a one-term exponential and linear model (Table A2.5). Large inflations were most prominent within Satyrinae, that also tended to

generally have more smaller body sizes. The largest species with vein ratio over 3 was *Ninguta schrenkii*, and had a body size index of 190.8. All species with vein ratios over 3 were below this body size score (Figure 3.16A). Both within Satyrinae and amongst other subfamilies, large inflations were most prominent in tribe Satyrini (Figure 3.16B, C). All species with more than one inflated vein fell within Satyrini (Figure 3.16D). Within Satyrini, there was more variation amongst size and vein ratio among species, although still demonstrating a two-term exponential relationship. Although this relationship was weaker than the analyses of all species and of Satyrinae, it was still stronger than linear or one-term exponential relationship (Table A2.5).

Tribe and subfamily were both found to have a significant effect on ratio, with Satyrinae having the most signifncant effect on ratio (Table A2.6). However, when analyzing only within Satyrinae, Satyrinae-specific tribes did not have a significant effect on vein ratio (Table A2.6). Within eared species, subfamily and tribe did not have a significant effect on vein ratio (Table 2.6).

Amongst all species, those with well-developed ears varied in vein size ratio. Species with uncertain or no ears tended to have small to medium body sizes and small vein ratios (Figure 3.17A). Amongst species that have ears, most belonged to the subfamily Satyrinae, and species demonstrated a best relationship fit using a two-term exponential model (Linear: R^2=0.4059, Log: R^2=0.4387) (Figure 3.17B). All species with large wing veins (>3:1) were in the Satyrinae (except for the one Biblidinae sampled), and tended to be of smaller body size index (Figure 3.17B). All large species, with body size index over 962.5, happened to be in Satyrinae as well, and had vein ratios under 3. Amongst the species I sampled for the other subfamilies with ears, Apaturinae,

Biblidinae, and Charaxinae, all had vein ratio under 3, except for the one Biblidinae species, that had vein ratio of 4.436 (Table 3.1, Figure 3.17B). Satyrinae species that have ears, which is all except for *Zethera musa* (Zetherini) and *Paralethe dendrophilus* (Dirini), demonstrated a best relationship fit using a two-term exponential model (Linear: R^2=0.3892, Log: R^2=0.4491). Eared Satyrinae species with smaller body sizes and larger vein ratios were prominently belonging to the tribe Satyrini. Eared Satyrinae species that had larger body size with small vein ratios were all Brassolini and Morphini species (Figure 3.17C). Overall, these results demonstrate that smaller sized species tend to have relatively larger size veins, and tend to also be within subfamily Satyrinae and tribe Satyrini.

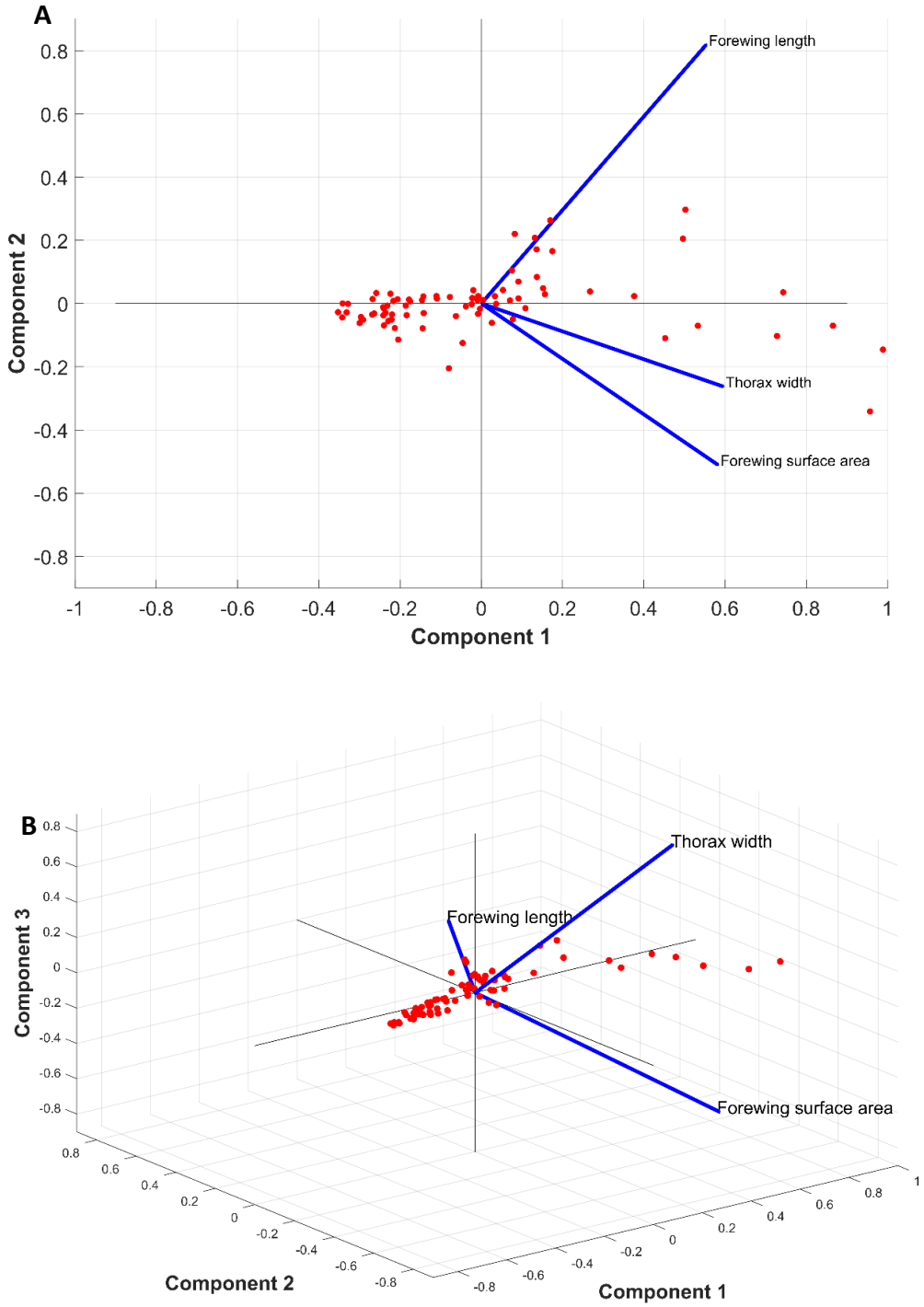
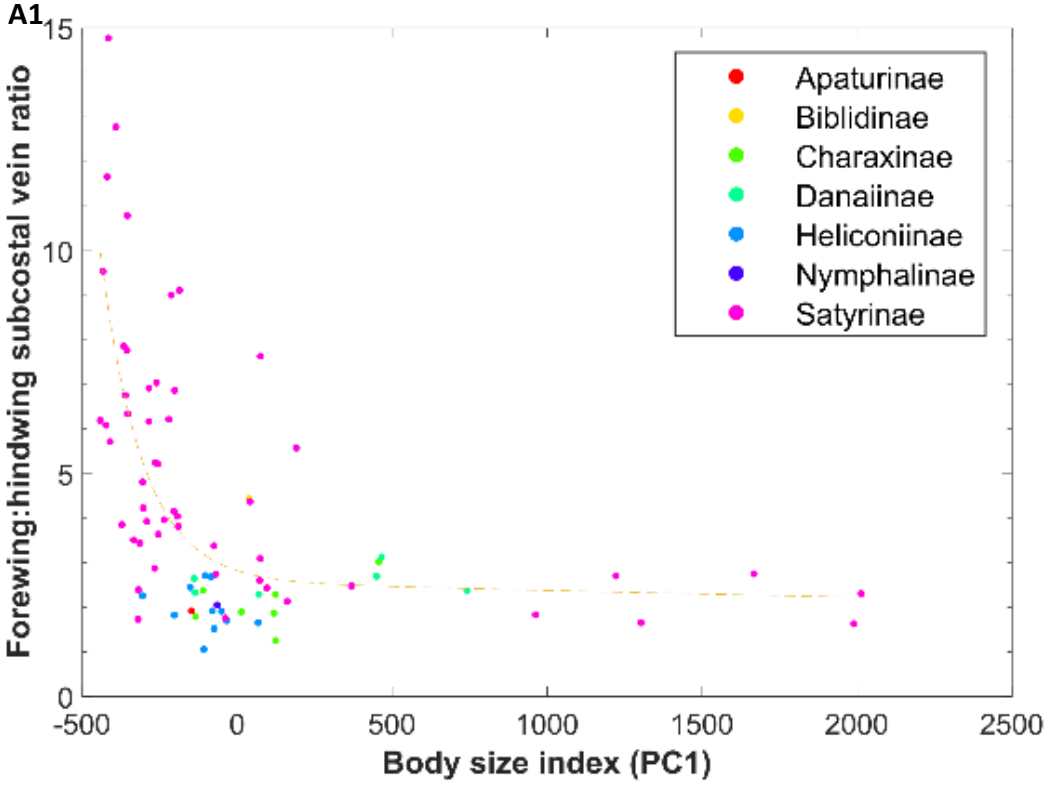
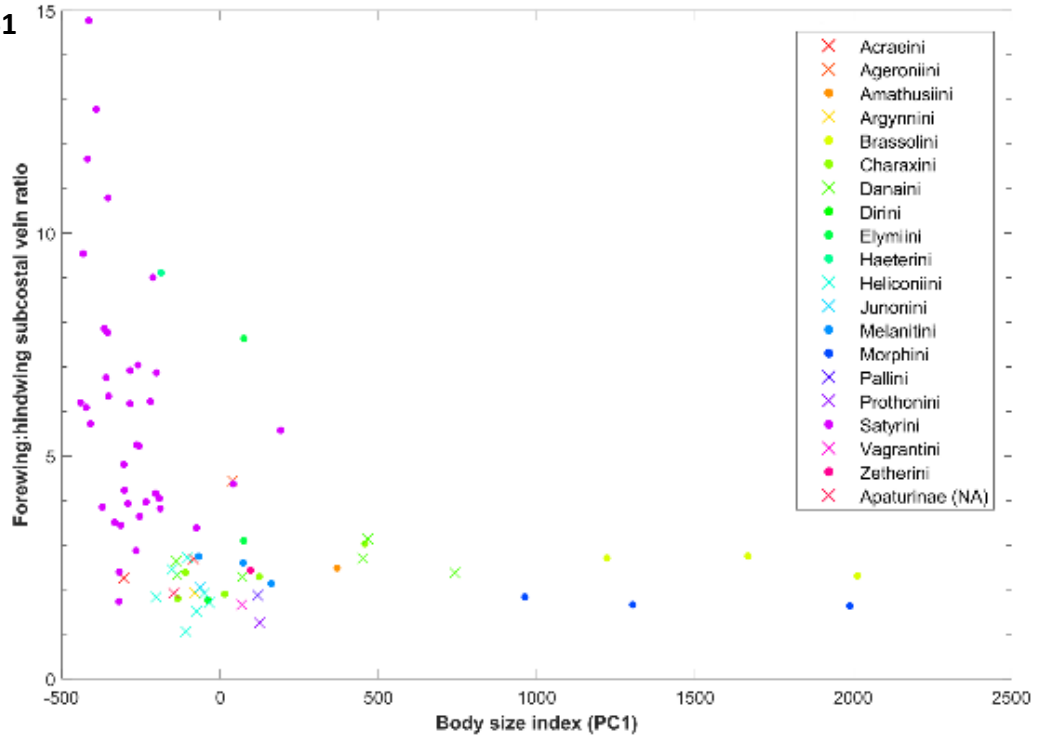


Figure 3.15. (A) PCA plot of trait loadings, on PC1 vs PC2, created from right-forewing length, right-forewing surface area, and thorax width. (B) PCA plot of trait loadings, on PC1, PC2, and PC3, created from right-forewing length, right-forewing surface are, and thorax width.

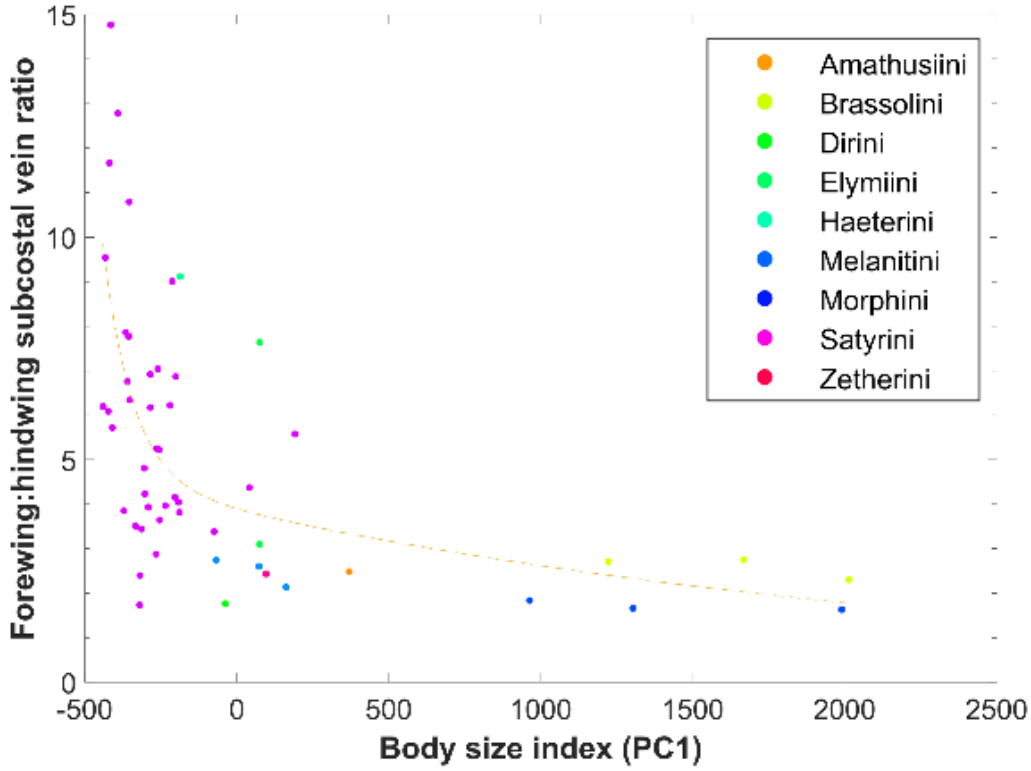
A1


Forewing:hindwing subcostal vein ratio
Body size index (PC1)
Apaturinae
Biblidinae
Charaxinae
Danaiinae
Heliconiinae
Nymphalinae
Satyrinae

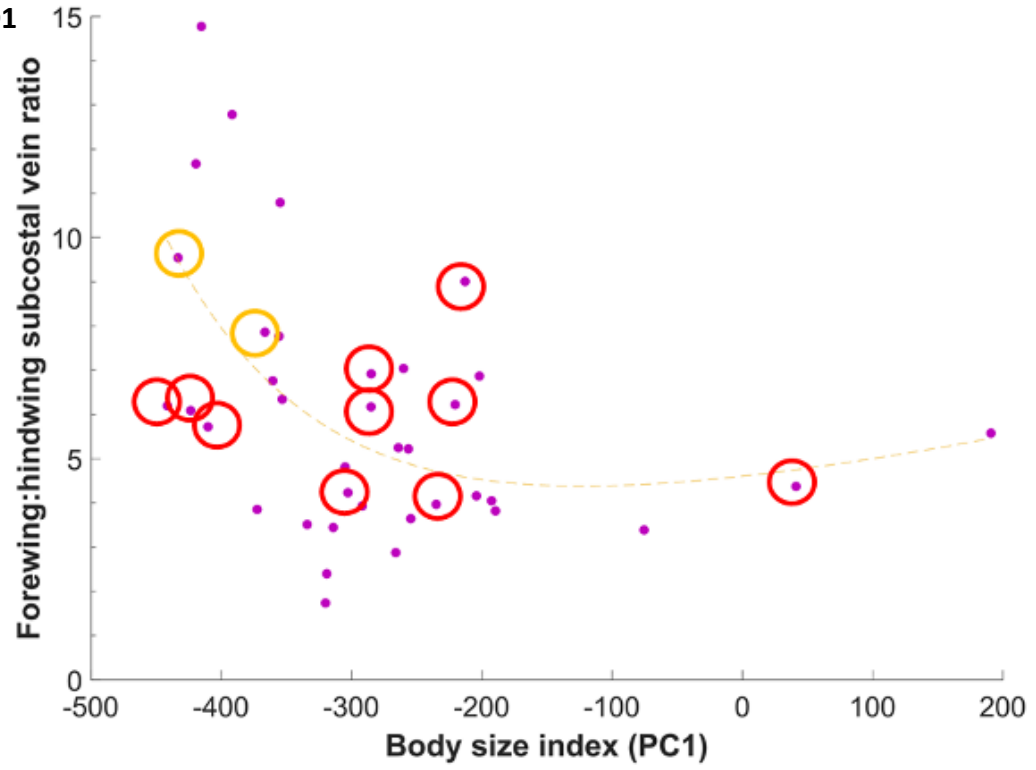
B1


Forewing:hindwing subcostal vein ratio
Body size index (PC1)
Acraeini
Ageroniini
Amathusiini
Argynnini
Brassolini
Charaxini
Danaini
Dirini
Elymiini
Haeterini
Heliconiini
Junonini
Melanitini
Morphini
Pallini
Prothonini
Satyrini
Vagrantini
Zetherini
Apaturinae (NA)

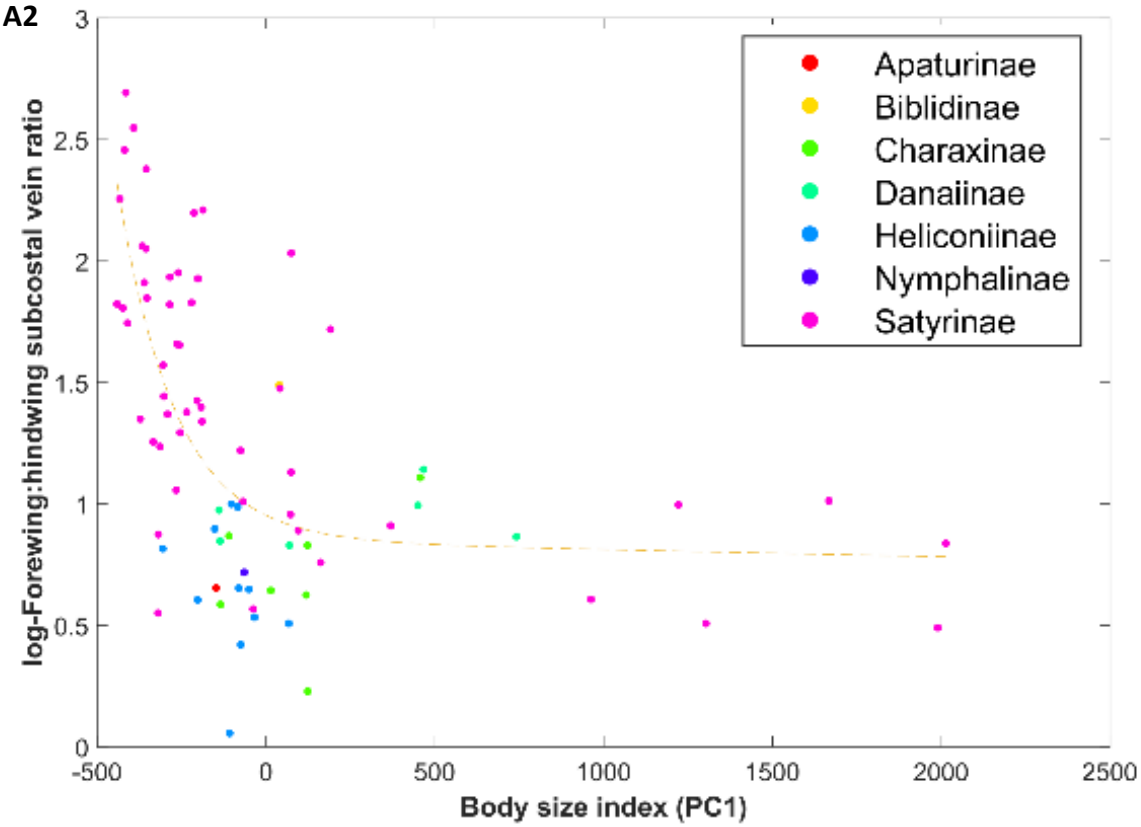
C1


Amathusiini
Brassolini
Dirini
Elymiini
Haeterini
Melanitini
Morphini
Satyrini
Zetherini
Forewing:hindwing subcostal vein ratio
Body size index (PC1)
0
5
10
15
-500
0
500
1000
1500
2000
2500

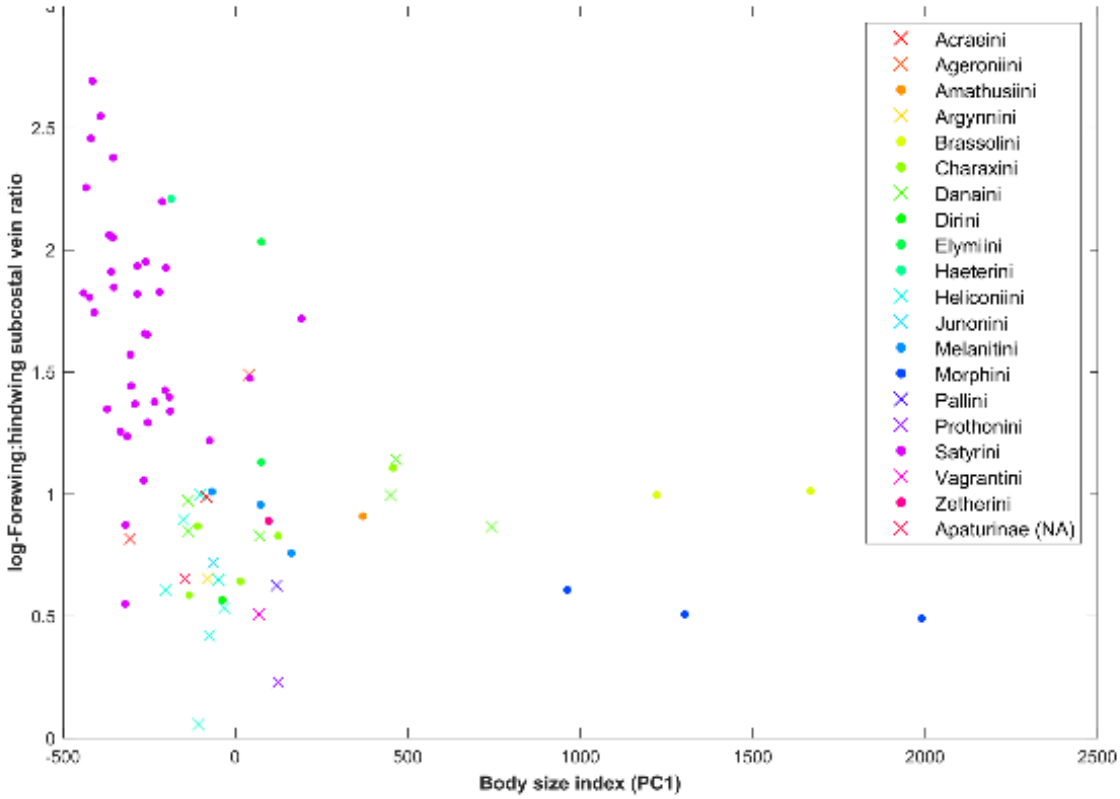
D1


Forewing:hindwing subcostal vein ratio
Body size index (PC1)
0
5
10
15
-500
-400
-300
-200
-100
0
100
200

A2


log-Forewing:hindwing subcostal vein ratio
Body size index (PC1)
Apaturinae
Biblidinae
Charaxinae
Danaiinae
Heliconiinae
Nymphalinae
Satyrinae

B2


log-Forewing:hindwing subcostal vein ratio
Body size index (PC1)
Acraeini
Ageroniini
Amathusiini
Argynnini
Brassolini
Charaxini
Danaini
Dirini
Elymiini
Haeterini
Heliconiini
Junonini
Melanitini
Morphini
Pallini
Prothonini
Satyrini
Vagrantini
Zetherini
Apaturinae (NA)

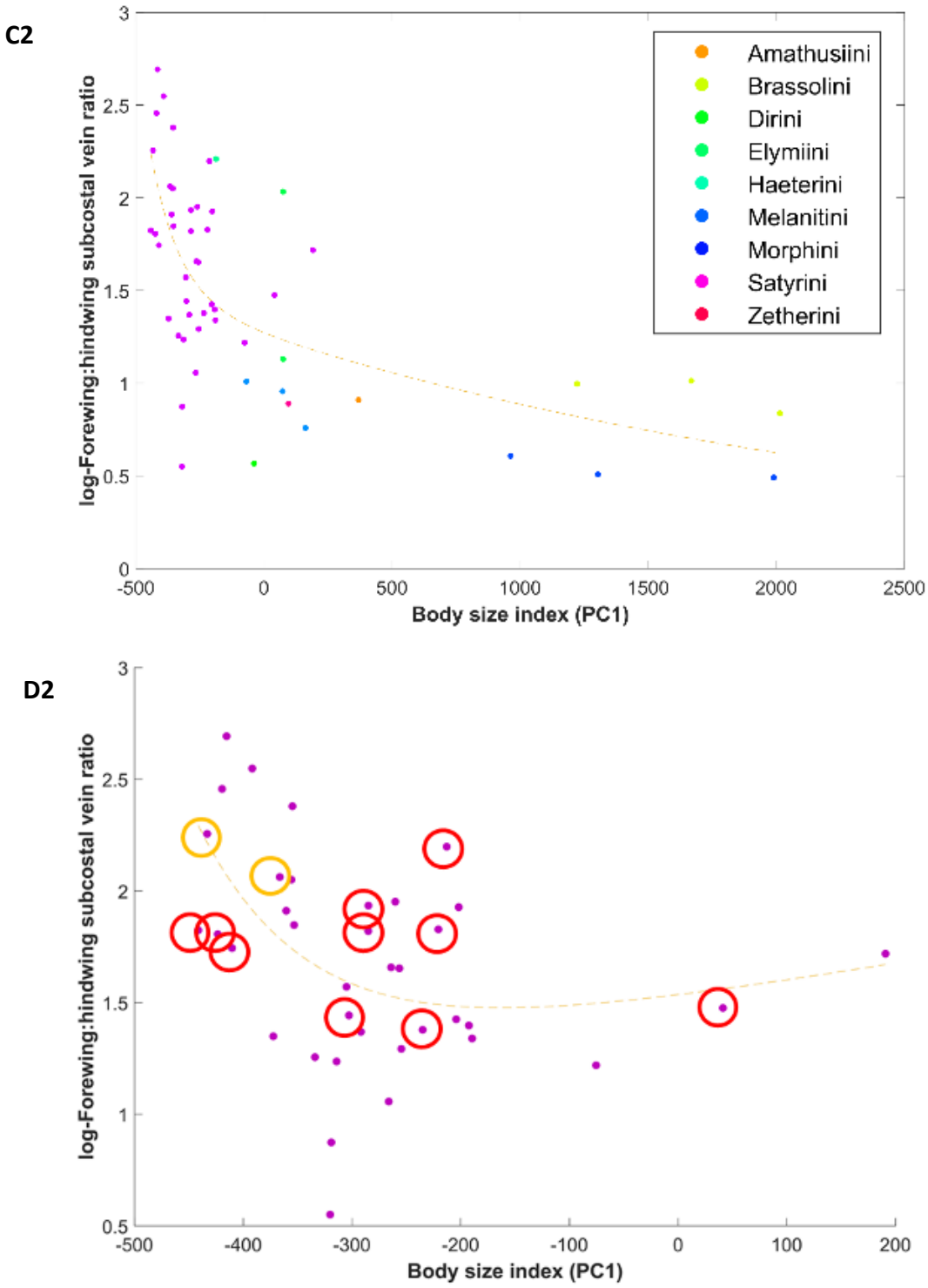
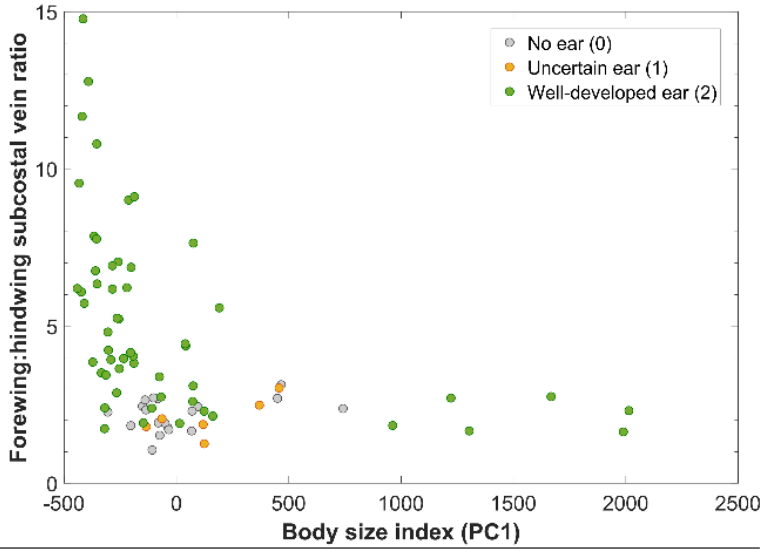
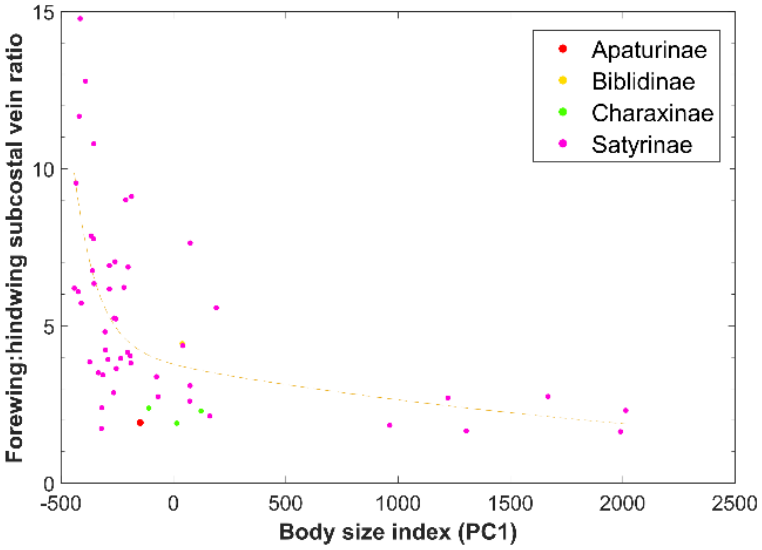


Figure 3.16. Relationship between body size (PC1) and subcostal forewing:hindwing vein ratio (ratio method 1). Top (1): Linear plots. Bottom (2): Log-transformed plots. Dashed pale yellow line in A, C, D = 2-term exponential fit. (A) All species sampled, sorted by Subfamily. (B) All species sampled, sorted by Tribe. Tribes denoted by X: Tribes outside Satyrinae. (C) Satyrinae species sampled, sorted by Tribe. (D) Satyrini species sampled. Red circles: Species with enlarged Cubital and Anal veins as well, yellow circles: Species with enlarged Cubital vein as well (Table 3.1).

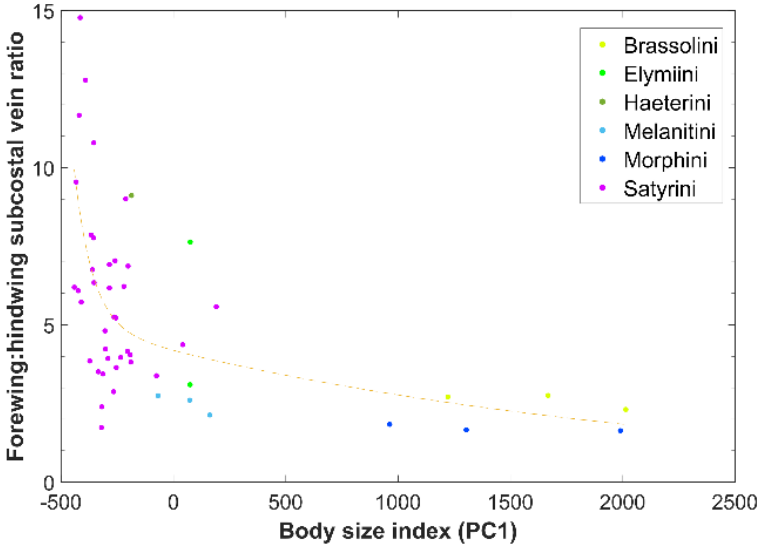
A1


No ear (0)
Uncertain ear (1)
Well-developed ear (2)
Forewing:hindwing subcostal vein ratio
Body size index (PC1)
0
5
10
15
-500
0
500
1000
1500
2000
2500

B1


Apaturinae
Biblidinae
Charaxinae
Satyrinae
Forewing:hindwing subcostal vein ratio
Body size index (PC1)
0
5
10
15
-500
0
500
1000
1500
2000
2500

C1


Brassolini
Elymiini
Haeterini
Melanitini
Morphini
Satyrini
Forewing:hindwing subcostal vein ratio
Body size index (PC1)
0
5
10
15
-500
0
500
1000
1500
2000
2500

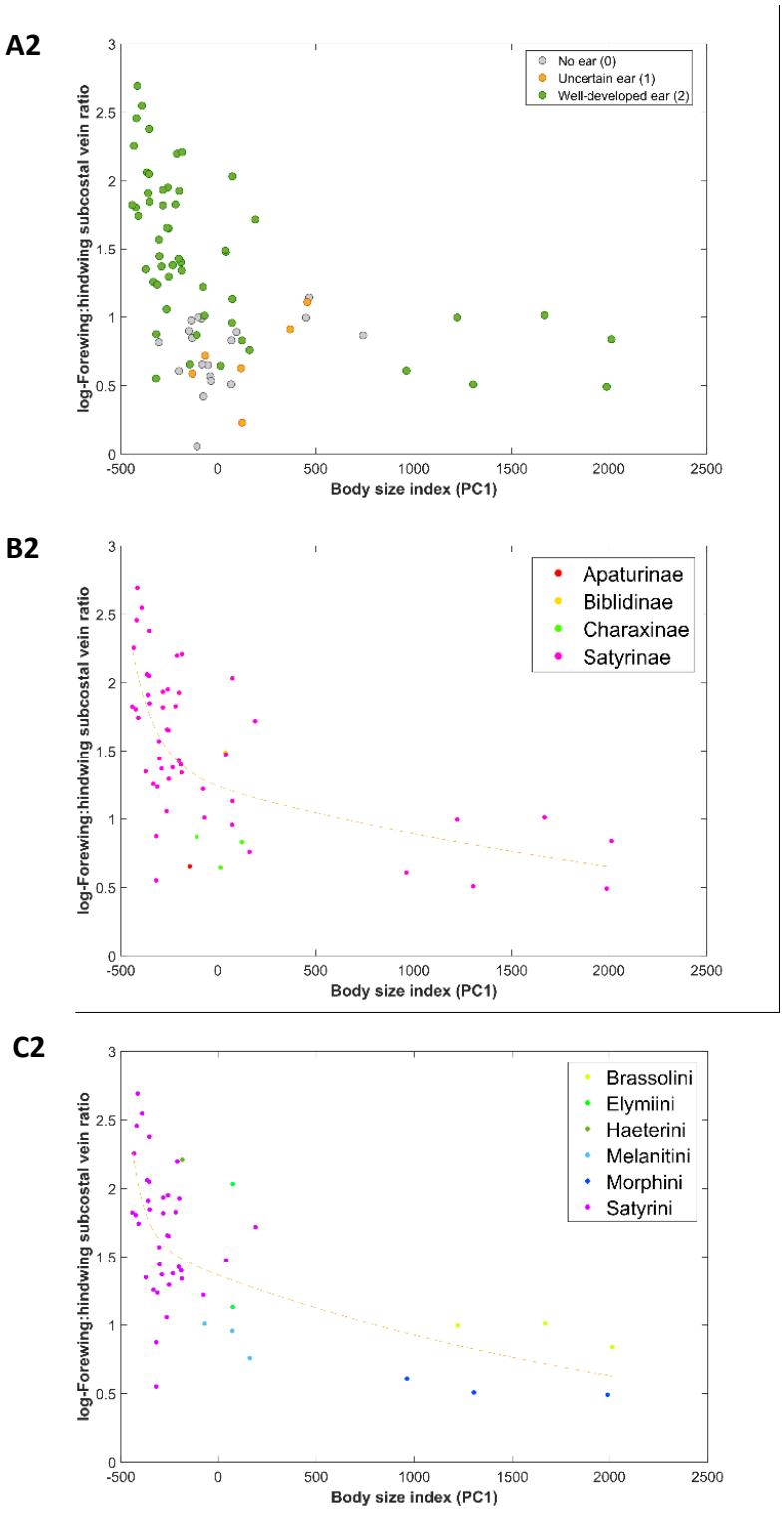


Figure 3.17. Relationship between body size (PC1) and vein size, using subcostal forewing:hindwing vein ratio (ratio method 1). Left: Linear plots, right: Log-transformed plots. (A) All species sampled, sorted by ear state, (B) Only eared species (level 2 ears), sorted by subfamily. Dashed pale yellow line = 2-term exponential fit. (C) Only Satyrinae species, with ears (level 2 ears), sorted by tribe. Dashed pale yellow line = 2-term exponential fit.

3.5. Discussion

3.5.1. What is a Wing Vein Inflation?

A vein inflation is a conspicuously enlarged wing vein that has been historically and informally used as a distinguishing characteristic of Satyrinae. The inflated vein was first acknowledged and drawn by le Cerf (1926) who studied it only in relation to studying the VO, mentioning how the cuticle of the VO occurs at the end of the rounded protuberance of the vein. Ford (1945) notes that "one or more of the nervures on the forewings are dilated at the base, and this is a distinguishing feature of the family [Satyridae – now treated as subfamily Satyrinae]". Comstock (1949) echoes this statement, saying "Satyrinae: With some of the veins of the forewings greatly swollen at the base", as does Miller (1968), saying "the inflation of the forewing veins has long been considered a key characteristic of the Satyridae." The veins are described as a "very thick area in the subcostal vein of the forewings" (Devries, 1987). Ackery et al. 1998 notes that "traditionally, [Satyrinae] has been defined by presence of swollen veins at the forewing base" and "although most do have one or more of these veins", some included genera do not (e.g. *Melanitis, Lethe*), while the character recurs in other Nymphalidae (e.g. *Maestra*, *Ariadne*, etc.)". This quote most accurately reflects what is discovered so far based on more recent studies, including this one. Reference to "*Maestra, Ariadne*" refer to genera of the subfamily Biblidinae, which other studies have also noted enlarged wing veins occur (Jenkins, 1983; Monge-Najera et al. 1998). Monge-Najera and Hernandez (1991) describe the veins in sound-producing butterflies of the genus *Hamadryas*, as "highly swollen, reaching a diameter of 2-3 times that of the equivalent vein of silent species". Other studies also casually note the presence of the inflated vein in descriptions

of species, especially Satyrinae species; Murillo-Hiller (2006) describes the widest part of the swollen area in [*E. castrensis*'s] subcostal vein is 800 μm, about eight times the 100 μm width of the vein's base, and the length of the swollen area is 6000 μm, 1/3 of the distance to the distal wing border" and Neild et al. 2014 describes *Eupytchia alacristata*'s wing venation as "Most of forewing subcostal vein swollen; base of cubitus barely so." Freitas & Brown (2004) describe "inflate costal veins" as being part of a Biinae, now a genus absorbed in Charaxinae. This is most likely to be a typo however, as costal veins have never been seen in any species studied so far or in any other literature.

Thus, although there is reference and some measurements of the trait in previous literature, none of these studies have formally defined or characterized what is and is not an inflated vein. The first attempt to do so was by a previous student in the Yack lab, Laura Hall (2014). She sampled any butterfly species available to her, with a focus on Nymphalidae, and quantified the size of the vein inflation based on the ratio of the forewing vein to its respective hindwing vein. She chose the forewing "because there is no ear associated with the hindwing". Based on plots and visual observations, she found that all non-eared species did not display vein inflations and were all under the ratio of 3:1. Thus, she concluded that a FW:HW ratio greater than 3 is to be considered inflated whereas a ratio below 3 is to be considered non-inflated. In this current study, I attempted to characterize further wing vein inflations by testing more species, and using 2 general types of measurements, (1) FW: HW vein ratio, as performed by Hall (2014), and (2) FW: Reference vein ratio, to check for agreement. Ratio method 2 also ensures that the measurements come from the same wing (the forewing), to minimize possible effects of the hindwing developing differently than the forewing. Both methods resulted in wide

range of variation between species, even amongst those with higher ratios. However, the two ratio methods were strongly correlated, so in the body of my thesis I mainly used ratio method 1 to describe the range of 'inflations', and to further categorize them into inflated, not, or uncertain, for purposes of correlating to ear development and taxa (see discussion of these in the next sections). Both methods also included species that have large veins that are more like thick bases rather than inflations. These are distinct from "classic" large inflated veins, which are hollower and have a more distinct elliptical shape. This explains why we recommended *Danaus cleophile, Archaeaprepon demophon*, and *Panacea prola,* as "thick" type veins using ratio method 1, and to not be considered vein inflations since they do not have the same elliptical shape, and their vein is structured as solid cuticle. Conversely, this also explains why *Satyrodes appalachia* and *Oeneis polixenes* were classified as large despite being a ratio under 3; they were more elliptical-shaped and passed independent viewer assessment.

It is important to note that without clearly understanding the important morphological properties imparting function to these structures, it is difficult at this time to make a clear distinction between an inflated and non-inflated vein, as we would, for example, for an ear or no ear, a leg or not a leg, a wing or not a wing. At this point, it is inconclusive if there is a cut-off ratio to quantify a vein as an inflation, especially since relatively smaller elliptical shaped veins may be included in what qualifies as a vein inflation because they may function in hearing, while other larger veins may not. One approach to characterizing an inflation would be to use morphometrics, which incorporates variations in shapes, using landmarks, and quantifies a morphologically distinct trait or identifies a morphologically distinct group based on that variation. This

has been performed throughout literature, such as to classify snake eyes and eyespots in caterpillars (Hossie & Sherratt, 2014), mosquito wing shape types (Rohlf, 2002), and wing venation between sibling species in *Eubazus* wasps (Villemant et al. 2007). Combining ratios with morphometrics may also be performed, in which multivariate morphometry may determine best ratios for separating two or more groups, such as for discriminating two groups of parasitic wasp species (Baur & Leuenberger, 2011). Regardless, geometric morphometrics are easier to apply when one has a reference for an established structure, such as the features of a known eye being used to quantify an eyespot (Hossie & Sherratt, 2014). Thus, the physiological work must still be done to establish what degree of 'inflation' is considered 'functional', and to clearly distinguish between categories. At this point, the ratio methods and vein type categories I have used are only suggestions and working definitions. I suggest future quantification methods should focus on incorporating and adding stronger consideration to function and specific categorical features, such as shape (elliptical-shaped) and hollowness (to be involved in hearing), in combination with geometric or multivariate morphometrics, into what qualifies as a vein inflation. As more species are sampled and alternative methods of quantifying this trait are solidified, a more formal quantification of a vein inflation will be distinguished over veins that are simply large. For purposes of relating to the taxonomic distribution and ear states, in this study I present veins as inflated, not, or uncertain, in which an inflation meets the criteria of being a ratio larger than 3, with an elliptical shape, and confirmation by independent observer(s). Note that these are working classifications that will likely need to be revised upon further functional tests of veins, and more formal quantification of vein sizes.

3.5.2. Are Inflated Wing Veins Associated with Hearing?

One hypothesis tested in this thesis is that inflated wing veins are associated with hearing. The predictions for this hypothesis are: (1) they are associated with Vogel's organ, the ear of the butterfly, (2) ablating the wing vein impacts the tuning properties of the ear's membrane, and (3) inflated wing veins only occur in species that have ears. Predictions 2 and 3 were tested and supported by experimental evidence in Chapter 2. In Chapter 2, I proposed these large veins are adaptations for compensating or enhancing hearing for certain species that can hear, but not necessary or needed for all species that hear. However, these experimental results are only for one species. The 3rd prediction posed in this chapter, Chapter 3, is that species with inflated veins also always have ears. This study has provided support for this prediction: all species sampled that had inflated veins also had well-developed ears. However, not all butterflies with ears have large vein inflations. Further discussion of the taxonomic distribution of these inflations will be discussed in subsequent sections of this discussion.

As suggested in Chapter 2, vein inflations may enhance hearing via impedance matching and/or as a Helmholtz resonator. In impedance matching, the volume of air from the inflated vein behind the ear provides a pressure gradient against the volume of air behind the ear, allowing the ear to respond to low frequencies, which would otherwise cancel out without the pressure gradient. When sound pushes on the ear's volume of air, the inflated vein's volume of air produced a reactive force, resulting in a net displacement of ear's membrane, which allows for lower frequencies to be detected. Besides lacewings and katydids, which may use impedance conversion between air and fluid for hearing

(Michelsen, 1979; Monteleagre-Z & Robert, 2015), no other insect has an equivalent or similar accessory structure, in either form or function, to our knowledge. in which Alternatively, or additionally, I suggested the vein may be acting as a Helmholtz resonator, a cylindrical air space that would amplify pressure within the cavity, causing increased sensitivity and extended duration for low frequencies (Hartley & Stephen, 1992). This may also be possible due to the vein's shape and the ear's hearing properties (Chapter 2). This is seen in tettigoniids (*Hemisaga*), having slit cavities associated with their tympanal ears, acting as coupled Helmholtz resonators (Hartley & Stephen, 1992), and horseshoe bats, whose inflated nasal cavities may act as Helmholtz resonators, shown by increased sound sensitivity in response to a larger pressure within the nasal cavity tracheal chambers (Suthers et al. 1988).

Other morphological features were noted, but not formally characterized. These include internal structures and other inflated veins besides the subcostal vein. Internal structures fell into informal categories of being spiral, honeycomb, irregular, or none. Even within the same category, internal structures varied considerably in shape, structure, and network, especially for structures designated as irregular. If the species had internal structures, they occurred only in the subcostal vein, except for *Y. albida*, which also had spiral structures in their cubital and anal vein. Among species sampled in this study, all species with internal veins occurred in subfamily Satyrinae and tribe Satyrini. These internal structures may contribute to hearing. For example, the spiral structure may be attenuating certain frequencies or pressure levels of sounds, perhaps for predators that share the same niches as the butterflies that have them. It is well-known that gun silencers, which have an internal spiral structure, attenuate sound, up to 90% of pressure

and 20 dB of sound level (Rehman et al. 2011). The polygonal or honeycomb structure may be preventing sound transmission loss; there is experimental evidence that shows honeycomb sandwich structures in lightweight building panels improves noise transmission loss (Wen-Chao & Chung-Fai, 1998). Some species also exhibited inflated cubital and anal veins. Within this study, all species that had enlarged cubital and anal veins were in subfamily Satyrinae and tribe Satyrini, and cubital veins were never inflated without the subcostal vein also being inflated, and anal veins were never inflated without the subcostal and cubital veins also being inflated. Having internal structures and/or more than one inflated vein may be an additional adaptation for even smaller sized species to further enhance or contribute to hearing (see *3.5.4*). The role of the internal structures and multiple inflated veins would be a newly emerging idea to explore. Further sampling and experimental and comparative studies are encouraged for species with and without variants of inflated veins and internal structures, as well as 3-D modelling or geometric morphometrics of the structures and the veins.

3.5.3. Taxonomic Distribution of Inflated Wing Veins

Previous taxonomic literature stated that "inflated wing veins" were a distinguishing character of Satyrinae, although this literature is outdated and excluded some tribes like Brassolini and Morphini, which were later absorbed into Satyrinae. Thus, previous literature may have assumed as a distinguishing feature of the group of species within specifically Satyrini, and/or other sister tribes. Based on the groups used to categorize the veins in this study (large, small, or uncertain), this mainly supports the previous literature, as only Satyrinae, out of all subfamilies sampled, had large (inflated)

veins, and within Satyrinae, almost all (35/36) Satyrini species had the large veins. All species sampled in Elymiini and Haeterini also had large veins, but only 2 and 1 species, respectively were sampled, so results are inconclusive about these tribes. However, it may be assumed that at least Haeterini is assumed to be included as having this trait as a distinguishing feature, as Ackery et al. 1998 and Freitas & Brown (2004) includes it under Satyrinae. This overlaps well with the occurrence of ears, which are seen to occur in every specie sampled of Satyrini, Haeterini, and Elymiini (see *3.5.4.* below). Thus, it may be more apt to claim that these inflated wing veins may be more of a distinguishing characteristic of specific tribes in Satyrinae, particularly Satyrini, although more sampling is required.

Vein inflations seem to occur more extensively and exclusively in some taxa, specifically Satyrinae and Satyrini. This was further supported by the fact that tribe and subfamily had a significant effect on vein ratio when accounting for body size, especially for Satyrinae (though this result should be carefully considered, given the non-significant effect of tribe when only looking at Satyrinae, and there needs to be more sampling before it is completely conclusive). One explanation for this is Satyrinae species, particularly Satyrini species, are relatively small compared to other groups, such as in Biblidinae or Morphini or Brassolini (Figure 3.16, 3.17, Ackery et al. 1998; Layberry et al. 1998). This size explanation is discussed in the next section (See *3.5.4*). Furthermore, this trait may have developed more prominently amongst certain groups that have niche habitats or predators, in which they have enhanced their hearing to be sensitive to, such as bird flight sounds (Fournier et al. 2013) or geographical niche: For example, *C. pegala*, a model Satyrini with these inflated veins, hears low frequencies (<5 kHz). *C.*

pegala is a holarctic species (Layberry et al. 1998), while *M. peleides* and *C. eurilochus* are neotropical, so adaptive pressures from different predators or surroundings would vary. *Cercyonis pegala* also tends to avoid disturbance, so perhaps it does not handle flooding and heavy rains as well as a neotropical species might (Hogsden & Hutchinson, 2004), suggesting differences in how species would listen to weather. The trait may have also resulted from Satyrini experiencing rapid, or adaptive radiation, which is supported based on their phylogenetic relationships (Pena, 2006). It is also worthy to note that species with more than one enlarged vein were in tribe Satyrini, and all have even smaller body size, with body size index of at or less than 35.157 (*Bicyclus sebetus*). Thus, although enlarged veins are purported to enhance hearing in species that have them, they do not occur in all species that have ears. The trait may have become a specialized accessory structure for relatively smaller butterflies. I recommend more sampling within Satyrinae and in outgroups, and I recommend the next set of analyses should account for phylogenetic bias, to incorporate these evolutionary relationships among species. I recommend phylogenetic generalized least square (PGLS) analyses to test the relationship between body size index and vein inflation ratio and using the most recently robust and resolved phylogenies for Nymphalidae (Wahlberg et al. 2006; Espeland et al. 2018), for Satyrinae (Wahlberg et al. 2009), and for within Satyrini (Pena et al. 2011). I recommend testing with Brownian model of trait evolution (same as independent contrasts) and/or with OU + Brownian model of trait evolution, and to test for phylogenetic signal, which will determine the tendency of related species to resemble each other, for this trait, the inflated vein (e.g. Thiagavel et al. 2018; Kang et al. 2017).

3.5.4. Why don't all butterflies with ears have inflated wing veins?

In chapter 2 it was hypothesized that inflated veins are largest in smaller species. The preliminary results from this study support this. Amongst all species, large veins were most prominent within Satyrinae, that also tended to have smaller body sizes. Both within Satyrinae and compared to other subfamilies, large inflations were most prominent in tribe Satyrini. Furthermore, all species with more than one inflated vein were Satyrini. Although well-developed ears occurred throughout Nymphalidae, most species within Satyrinae sampled had ears and all Satyrini sampled had ears, and all of these were comparatively smaller in body size. Eared Satyrinae species that had large body size with small vein ratios were Brassolini and Morphini species, which did not have inflated veins. Thus, although inflated veins seem to be closely associated with ears, not all species with ears have them. They may have evolved as an adaptation for relatively smaller species. Smaller insects face difficulty with sound detection, localization, and directional hearing, due to physical restraints in detecting low frequencies among background interference, especially from farther distances, and deriving enough interaural intensity and time differences between both ears (Bennet-Clark, 1998; Schmidt & Romer, 2013). Thus, these inflated veins may have evolved as an adaptation for relatively smaller butterflies, functioning as 'hearing aids' to overcome the challenge of hearing low frequency sounds.

Other small insects also overcome physical challenges with hearing using adaptations. Field crickets listen to a wide range of frequencies, including low frequencies around 4-5 kHz (Michelsen & Larson, 2008). Field crickets have a complex and modified system of trachea that they have adapted to conduct sound and enhance

directionality. By performing a comparative study among 40 species of crickets, it was found that smaller crickets compensated for their unfavourable (wavelength to sound) size for hearing by having relatively larger acoustic vesicles (Schmidt & Romer, 2013). As mentioned before, the parasitoid fly *Ormia ochracea* has a flexible bridge-like structure between its two ears that bends to localize and amplify low sound frequency vibrations, which especially helps it localize its host, the cricket (Robert et al. 1994, 1996), and tettigoniids (*Hemisaga*) have slit cavities associated with their tympanal ears that act as Helmholtz resonators, helping with increased sensitivity and extended duration of low frequencies (Hartley & Stephen, 1992). I propose that the inflated vein in butterflies provides an advantage to small butterflies in detecting and hearing low frequency sounds. It is a unique structure that occurs only in butterflies with ears but shows variation between species that do have ears. More work should be done using laser vibrometry and neurophysiology trials to test hypotheses on the functional significance of variation in this trait.

3.5.5. Conclusions

Inflated veins have long been described in the literature and used as taxonomic characters, but they have not been characterized, nor has their taxonomic distribution or function been studied. This study showed that inflated veins are associated with hearing organs, provided preliminary data to categorize inflations, and provided explanations for possible selection pressures, particularly body size, resulted in the evolution of these structures. Future work should include a more extensive phylogenetic survey within the Satyrinae to understand the evolution, in addition to laser vibrometry and

neurophysiology experiments to test hypotheses on the functional significance of wing vein variation. Comprehensive quantification of the vein is encouraged as well, such as through 3-D modelling or geometric morphometrics.

Chapter 4. General Discussion and Conclusions

4.1. Introduction

Butterflies have been well-studied for their visual and chemical sensory ecology, but relatively less is known about their sense of hearing, especially amongst diurnal butterflies. Even less is known about potential accessory structures in butterflies involved with hearing. This thesis provides an overview of hearing related morphology in butterflies within Nymphalidae, with a specific focus on the inflated vein trait, a structure that has been only informally noted in previous literature. I tested the 'hearing aid' hypothesis that inflated wing veins are involved with hearing, predicting that (1) they are associated with Vogel's organ, the ear of the butterfly, (2) ablating the wing vein impacts the tuning properties of the ear's membrane, and (3) they only occur in species with ears. I also tested the 'size' hypothesis that inflated veins are more prominent in smaller sized species, predicting that (1) they are more developed in smaller species, showing a negative correlation with body size and (2) that smaller species will more often have more than one inflated wing vein.

4.2. Hearing in butterflies

Although several groups within Nymphalidae are noted to include Vogel's organ (Freitas & Brown, 2004), few species have had their hearing or acoustic behaviour formally characterized. *Hamadryas feronia* (Biblidinae) (Yack et al. 2000), *Caligo eurilochus* (Lucas et al. 2014), and *Morpho peleides* (Lane et al. 2008; Mikhail et al. 2018) are tuned to sound frequencies under 4 kHz, based on neurophysiological or laser

vibrometer studies. *Erebia manto* and *E. euryale* (Ribaric & Gogola, 1996) have been determined to behaviourally respond to frequencies between 125 Hz to 16 kHz. Beyond these species, no other diurnal butterfly species have been formally tested experimentally or behaviourally until now. In Chapter 2, tuning properties of the ear's membrane in *Cercyonis pegala* (Satyrinae: Satyrini) were characterized for the first time. This species is one of the first Satyrini species (*Pararge aegeria:* Mahony, 2006), and the first model species with a representative inflated vein to have their hearing characterized. Although no neurophysiology trials were conducted for my study, the laser vibrometry data (chapter 2) provides strong support to demonstrate tuning and sensitivity of the hearing organ, due to replicability between laser vibrometry and neurophysiology trials for butterflies (Lucas et al. 2009; Lucas et al. 2014). The tympanal chamber is connected to the inflated vein, supporting the prediction that they are associated with each other. The frequency response of the ear's membrane vibrates like a drum around a single mode around 5 kHz, mode 1,1, and can be modeled as a simple harmonic oscillator. The membrane also vibrates at an antinode, mode 1,2, at around 14 kHz. Overall, *C. pegala* was determined to respond to low sound frequencies (<5 kHz), with a mean resonance frequency of 7.78 kHz, but non-selectively. Their broadband and non-selective tuning pattern is unique among diurnal butterflies tested to date (Lucas et al. 2009; Lucas et al. 2014) and even in other insects, such as crickets (Michelsen et al. 1994; Mhatre et al. 2011), that hear more selectively and tuned to specific frequencies.

The function(s) of hearing in mute diurnal butterflies remains unconfirmed. Butterfly hearing most likely functions in detecting predators, particularly birds, but this needs to be further formally tested. Flight sounds of black-capped chickadees, Eastern

phoebes, and blue jays, that find *C. pegala* particularly palatable (Bowers & Wiernasz, 1979), are broadband (100 Hz-20 kHz) with dominant frequencies around 1 kHz (Fournier, 2011; Fournier et al. 2013). The frequencies of the "frustration call" (such as when in hunger), in adult blue jays varies between 1.2-1.5 kHz (Conant, 1972) and the house sparrow's song has peak frequencies ranging from 3-6 kHz (Henry & Lucas, 2009). These frequencies correspond with the mechanical response of *C. pegala*'s ear. An alternative hypothesis to listening for predators, this butterfly may be listening to other species, such as bees that often share the same food and nectar sources and pollinate many of the same flower species (Iftner et al. 1992; Manitoba Museum 2014). Depending on the species, foraging bees can create buzzes or wingbeat frequencies ranging between 100-400 Hz in fundamental frequencies (de Luca & Vallejo-Marin, 2013; Miller-Struttman et al. 2017), and sometimes harmonic frequencies (integer multiples of the fundamental frequency value caused by resonance of the vibrating exoskeleton), up to 2000 Hz (2 kHz), but they contain significantly less energy than the lower fundamental frequencies. These low frequencies would be detected by *C. pegala*. The results on tuning properties of *C. pegala* presented in this thesis have provided further understanding into sensory ecology of butterflies, especially for the tribe Satyrini, a tribe that has had limited testing for their hearing. After characterising the tuning properties of the ear, I experimentally tested the function of the inflated vein of this species, and comparatively determine every species with an enlarged vein also had an ear.

4.3. Inflated veins

Some butterfly species have been determined to have characteristically unusual enlarged wing veins at the base of their ventral forewing, deemed "inflated veins." Satyrinae have been characterized by having these veins as a distinguishing feature of the subfamily (Ford, 1945; Miller, 1968; Ackery et al. 1998), but until now their function has remained untested or inconclusive, and the trait was never formally mapped or given formal quantification.

Chapter 2 tested the function of the inflated vein experimentally for the first time, in a representative species *Cercyonis pegala* (Satyrinae: Satyrini). The inflated vein was determined to act as a "passive" hearing aid, enhancing low sound frequency sensitivity. Ablating the vein reduced sensitivity to lower frequencies: the ear's mean resonance frequency increased to 8.08 kHz, and significantly lowered the mean displacement at resonance frequencies and mean maximum displacement. Thus, the inflated vein of *C. pegala* may enhance hearing at mid-range frequencies such as 5-7 kHz, while maintaining sensitivity via broadband tuning for lower frequencies (1-5 kHz). These results supported my hypothesis that inflated wing veins are involved with hearing, by confirming the prediction that ablating the vein would have a significant effect on the tuning properties of the ear. Depending on configuration, the ear may act as a pressure receiver, a pressure gradient receiver, or a pressure difference receiver, all of which helps the insect hear differently (Michelsen & Larson, 2008). Then, if the ear's air space is connected to the swollen vein's air space, the vein can act in impedance matching. As discussed in Chapter 2, inflated veins most likely function via acoustic impedance matching. Air against air impedance has never been seen in any insect, but lacewings and

katydids may use impedance conversion between air and fluid for hearing (Michelsen, 1979; Monteleagre-Z & Robert, 2015). Alternatively, or additionally, inflated veins could function as Helmholtz resonators, in which the cylindrical air space of the inflated vein amplifies pressure within the cavity and allows for increased sensitivity and extended duration of low frequencies (Hartley & Stephen, 1992). This could be likely given the shape of the subcostal vein and the vibration data. This is seen in tettigoniids (*Hemisaga spp.*), having slit cavities associated with their tympanal ears, acting as coupled Helmholtz resonators (Hartley & Stephen, 1992), and horseshoe bats, whose inflated nasal cavities may act as Helmholtz resonators, shown by increased sound sensitivity in response to a larger pressure within the nasal cavity tracheal chambers (Suthers et al. 1988). Further testing could determine that the vein acts as a resonator, an amplifier, and/or assist with sound localization or directionality. For example, if the vein acts as a resonator, creating resonance in the vein, the connection to the ear can stimulate and change the mechanical stimulation of the tympanal membrane, creating higher sensitivity and tuning frequency, which has been seen in cicadas and crickets (Bennet-Clark, 1999). Thus, further investigation into modelling or experimenting of the inflated veins, such as through geometric morphometrics or forced pressure increases in the vein, is suggested to determine its acoustic mechanism and functions.

As discussed in Chapter 3, vein inflations may be a result from selection pressure to hear low frequencies, perhaps due to habitat or predator differences. One of the most promising explanations for the variation in occurrence of vein inflations is related to body size. The idea that the vein inflations enhance hearing may be especially true for smaller butterflies, which many Satyrinae butterflies are compared to butterflies in other

subfamilies. Smaller insects face difficulty with sound localization and directional hearing due to physical restraints in deriving sufficient interaural time and intensity differences between both ears (Bennet-Clark, 1998). These inflated veins may have adapted more prominently and more well-developed and serve a more functional purpose in relatively smaller butterflies, and this has been further supported by results in Chapter 3. Wing veins and body size traits were examined for 79 different species throughout Nymphalidae, representing 7/12 subfamilies. Of Satyrinae, species representing all tribes were examined. An exponential relationship was determined for every analysis between body size and wing vein size, in which smaller-sized butterflies had relatively larger vein sizes, with smaller butterflies being heavily distributed amongst Satyrinae and Satyrini species. All species with more than one inflated vein were also relatively small. Every butterfly classified to have a large vein also had an ear as well. These results support the hypotheses that inflated wing veins are involved with hearing and that inflated veins are more pronounced in smaller species, by confirming the predictions that every butterfly that has an enlarged wing vein also has an ear, and that wing veins are more well-developed in smaller-sized butterflies.

Some species also had geometric structures within their inflated veins. These internal structures may explain the unique flat and broadband hearing pattern *Cercyonis pegala* has over other diurnal butterflies within the same subfamily, which are determined to hear more selectively. *C. pegala* possesses internal structures within their subcostal vein, while *Morpho peleides* and *Caligo eurilochus* do not (personal observation), suggesting that the internal structures may further contribute to their

differences in hearing, albeit further investigation and characterization is required for these structures.

4.4. Conclusions

This thesis has provided experimental and comparative evidence for the function of the inflated vein, a trait that has been used for taxonomic purposes but only informally noted in the literature until now. My thesis makes the first attempt to characterize the morphology of these structures and to test a hypothesis on their function. My results provide strong support for the hypothesis that inflated veins function in hearing, and particular in Satyrini species, and in small butterflies. I also for the first time provide insight into membrane mechanics of the tympanal ear in a Satyrini species, *C. pegala*. Investigating the hearing is essential in further understanding sensory ecology of butterflies, many of which are endangered. Fifteen out of the 52 currently endangered or threatened Nymphalidae species are in the tribe Satyrini and purportedly have these inflated veins (IUCN, 2018). Butterflies serve as bioindicators, pollinators, and even symbols of beauty and importance for people (Liu 2001; Nabhan et al. 2006; Gehring & Bennett 2009; de Araújo et al. 2014). These inflated vein structures are a unique mechanism of auditory frequency tuning in insects, which may have implications for the development of novel acoustic technology and provide insight into the relatively little that is known about acoustic sensory ecology of butterflies. It seems these inflated veins would be a "sound investment" for not only the species who have developed them, but also for future biologists, acousticians, and innovators.

Appendix 1: Laser vibrometry supplementary material

A1.1. Materials and Methods

A1.1.1 Animals

Cercyonis pegala [nephele] were collected as adults from various locations near Ottawa and Perth, Ontario, Canada. Coordinates of collection sites: (45°10'28.2"N 76°02'48.8"W) (45°12'27.6"N 76°04'27.3"W) (44°43'58.4"N 76°42'08.4"W)

Sex was determined by genital morphology.

A1.1.2. Tympanal and wing vein morphology

Photographs were taken using a light microscope (Leica M205C) equipped with a camera (Leica DMC4500) and measurements of the tympanal membrane surface area made using Leica Application Suite (4.8.0) software. To image the tympanal membrane and the connectivity between the tympanal chamber and subcostal vein, six dried specimens were dissected, mounted on aluminum stubs, coated 60% gold/40% Palladium coating (Anatech Hummer VII) and imaged with a scanning electron microscope (Tescan, Model: VegaII XMU).

A1.1.3. Laser Vibrometry

Vibration measurements were made with a scanning laser Doppler vibrometer (Polytec PSV 400) coupled with an OFV-505 sensor fitted with a close-up unit using methods modified from (Mhatre et al. 2016). Butterflies were temporarily anesthetized

using CO_2 in order to mount and position them for scanning. Specimens were positioned right side up on a block of Blu-Tack® (Bostik, Chelsea, MA, USA) modelling clay, using wire staples to restrain the abdomen, and fastened onto a steel rod with a rotatable platform. The forewing was positioned overtop the hind wing to expose the tympanal region, and both wings were secured with strips of cardstock paper and Blu-Tack (Figure A1.1). A fringe of protective scales was removed with forceps and the tympanal membrane positioned perpendicular to the laser beam. Vibration measurements were acquired through the same NI DAQ PC 6110 board used for stimulus generation (see below). The experiment was isolated from vibrations using a vibration isolation table (Newport VH3036 W-OPT) and by placing it in an acoustic isolation booth (Eckel XHD-BATTEN). Animals were alive throughout the duration of the experiment as verified by antennal response when stimulated.

Vibrations of the tympanic membrane were measured in response to acoustic stimuli. For each butterfly, the scan area was set to include the entire ear membrane. Each scan, comprising ~185±30 measurement points placed on a square grid (mean ± SD, Figure 2.2a), took approximately 20 minutes. Methods used here are similar to those used previously to investigate the frequency response of tympanal membranes (e.g. Mhatre et al. 2016; Mhatre & Robert 2013). Acoustic stimuli were 160 ms periodic chirps containing frequencies ranging from 0.75 to 20 kHz with a frequency resolution of 6.25 Hz. All data were acquired and digitized at a sampling rate of 51.2 kHz. Stimuli were produced by the vibrometer software (Polytec Scanning vibrometer software version 9.1.2) through a National instruments DAQ board (PCI 6110), amplified and broadcast via a mylar membrane loudspeaker. The acoustic stimulation was free field and

continuously monitored. A Bruel and Kjaer 1/8th inch microphone (Type 4138) (frequency response ± 1 dB: 20 Hz to 80 kHz) was placed ~1 cm above the animal's tympanal membrane and connected to a Bruel and Kjaer pre-amplifier (Nexus 2690), the A/C output from which was continuously monitored as a reference by the vibrometer software (Figure A1.1.). The delivered sound stimulus was flat (± 3 dB) across the frequency range of interest. The average Fast Fourier Transform (FFT) amplitudes of the delivered stimuli were 20 mPa (60 dB SPL re 20 μPa). The vibrometer measured the velocity of the membrane at each point as it vibrated in response to acoustic stimulation. An FFT of this response is calculated using the same FFT parameters as used for the stimulus. From this data, other measures such as the displacement amplitude, phase and coherence can be calculated. The stimulus was presented 20 times at each point and the response of the membrane was averaged across all presentations using the complex averaging procedure, which accounts for both the magnitude and phase of the response. Magnitude of membrane displacement (nm/Pa), phase, and coherence of the response at each scan point were calculated from the vibrometer's measurements of the membrane's velocity at each point. Scan quality was ensured by monitoring coherence and ensuring a coherence level of at least 0.8 where significant motion was observed on the membrane. Video animations of the membrane vibration were created using the PSV software.

We first characterized the vibration properties of the tympanal membrane in each of seven male and seven female butterflies with intact subcostal veins. Following each scan, we then assessed how the inflated subcostal vein contributed to vibration properties of the tympanal membrane by ablating the intact subcostal vein. To do this, LDV scans were repeated after longitudinally cutting (ablating) ventral surface of the inflated

subcostal vein. This was done by dragging a teasing needle from the most posterior point of the vein inflation vertically down the length of the vein. A longitudinal cut was made to ensure that the vein would not reseal through healing. Ablation removed the ventral surface of the Sc vein, and disrupted the "honeycomb" internal structure, such that the remainder of the vein still left a closed wing surface, but the lumen of the vein was opened (Figure A1.2). We ensured that the tympanum, and the cubital and anal veins were not affected. Measurements were repeated as described above.

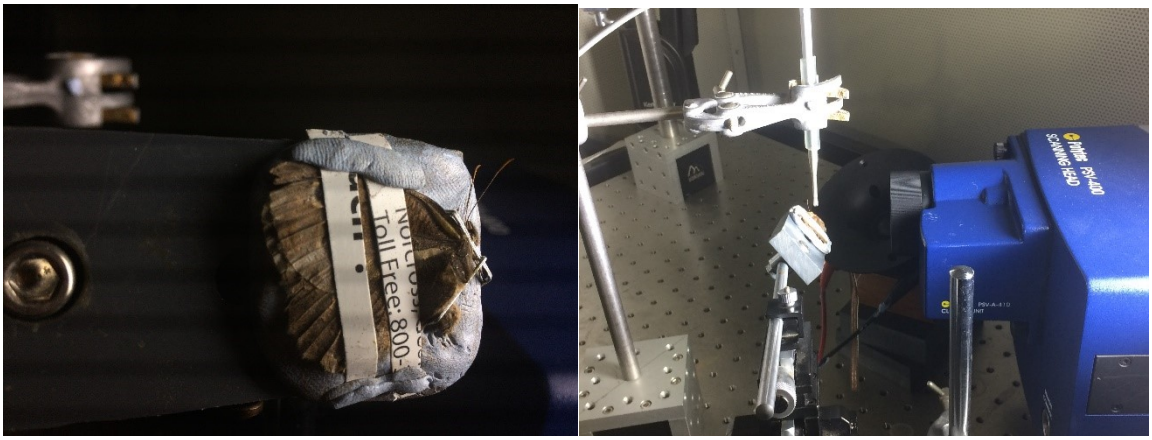


Figure A1.1. Experimental setup for laser vibrometry measurements. A: *Cercyonis pegala* specimen mounted on rotatable metal platform attached to a steel rod. B: Experimental set-up for laser vibrometry, with microphone above butterfly.

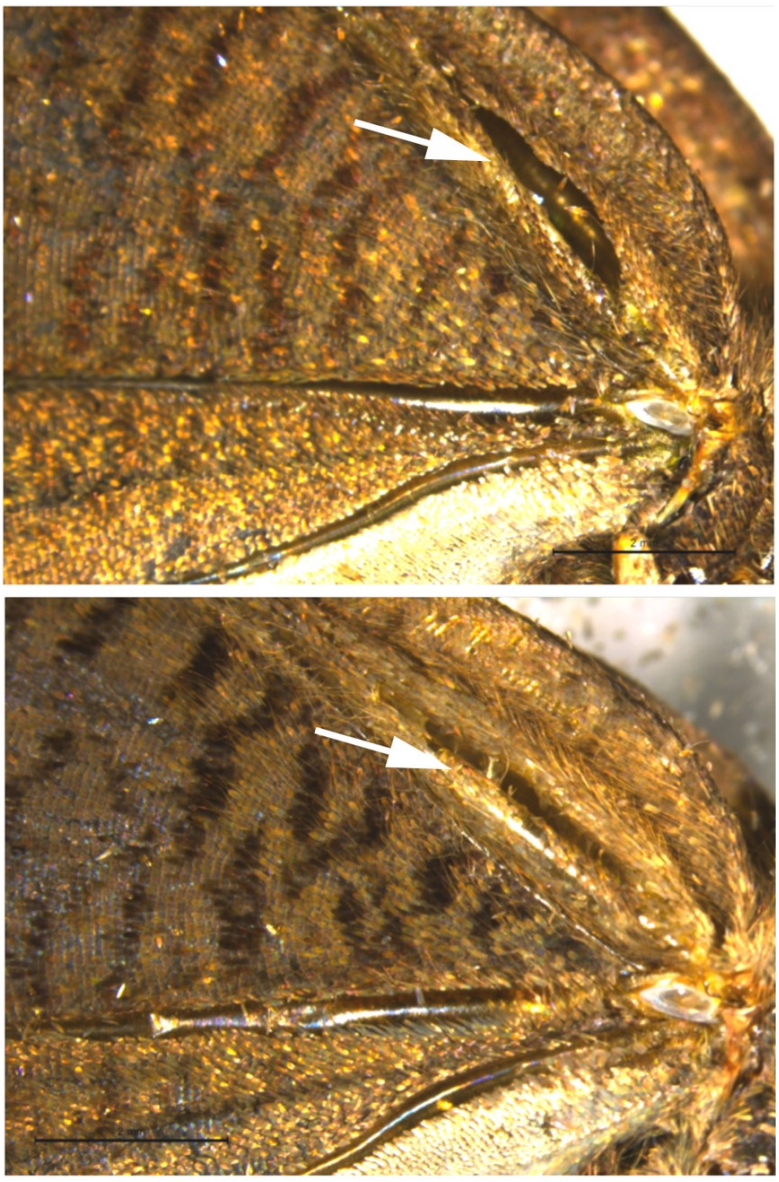


Figure A1.2. Ventral surface of forewing in *C. pegala* showing representative examples of longitudinal cuts of the Sc vein for ablation experiments. White arrows point to the cuts. Scale bars: 2 mm.

A1.2. Results

A1.2.1. Tympanal and Wing Vein Morphology

The subcostal (Sc) vein is the most obviously modified from "typical" vein structure (Figure A1.3). Its connection to the ear, and its large elliptical form and 'hollow' structure suggest an auditory function.

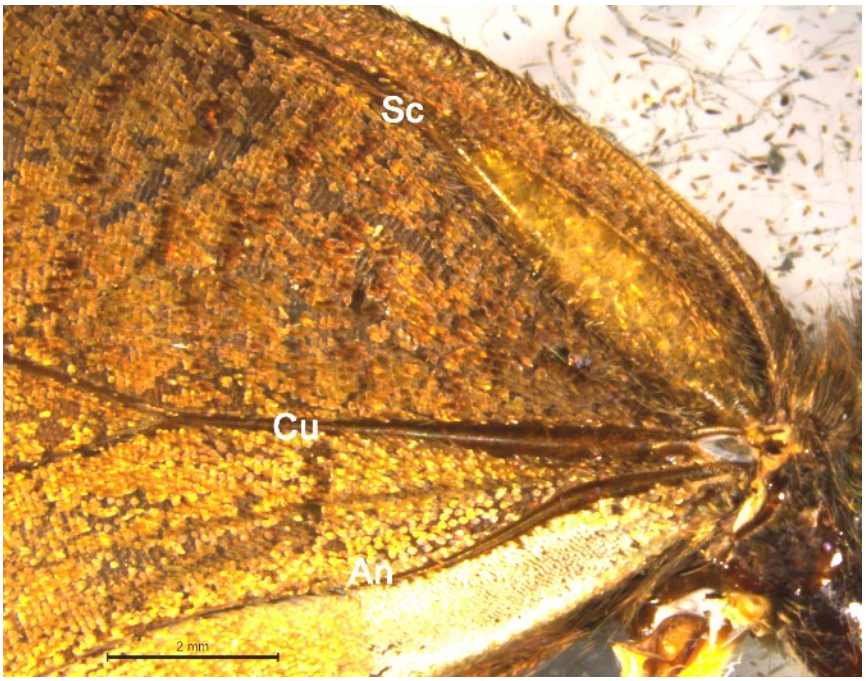


Figure A1.3. Ventral surface of right forewing in *C. pegala* showing the main wing veins. The subcostal vein (Sc) is clearly enlarged in comparison to the cubital (Cu) and anal (An) veins. Scale bar: 2 mm.

There was no significant difference between the surface area of the tympanal membrane between sexes ($p<0.05$), even though the forewing length (representing body size) exhibited significant sexual dimorphism ($p<0.05$) (Table A1.1).

Table A1.1. Morphology of Tympanal membrane in male and female *C. pegala.* [1]Sexual dimorphism between sexes for forewing length measured using unpaired t-test with unequal variances. [2]Sexual dimorphism between sexes for VO surface area measured using ANCOVA, accounting for forewing length as covariate.

Side	**Forewing length (mm)**		**p-value[1]**	**Tympanal membrane length (mm)**		**p-value[2]**	**Tympanal membrane width (mm)**		**p-value[2]**	**Tympanal membrane surface area (mm^2)**		**p-value[2]**
	Male	Female		Male	Female		Male	Female		Male	Female	
Left	24.4 ± 1.3	26.6 ± 2.9	**0.02898**	0.885 ± 0.040	0.919 ± 0.066	0.49	0.331 ± 0.042	0.374 ± 0.037	**0.017**	0.243 ± 0.040	0.274 ± 0.032	0.1917
Right	24.4 ± 1.1	26.9 ± 1.5	**0.00006**	0.850 ± 0.047	0.910 ± 0.064	0.11	0.342 ± 0.026	0.380 ± 0.035	0.3114	0.230 ± 0.031	0.281 ± 0.030	0.3243

A1.2.2. Laser vibrometry

No significant differences occurred between sexes for each tympanal behaviour parameter measured and calculated from laser vibrometry, for both intact and ablated conditions (Table A1.2.).

Male and female tympanal displacement is most responsive to low frequency sounds (<5kHz), demonstrating flat displacement below 5 kHz and falling steeply after 8 kHz (Figure A1.4a). Males exhibit a maximum displacement of 211 ± 105 nm/Pa and females exhibit a 196 ± 100 nm/Pa, with comparable resonance frequencies (Table A1.2.).

Upon ablation, both males and females demonstrate reduced sensitivity overall, showing erratic displacement from frequencies up to ~1.5 kHz, and reaching a maximum displacement of only 136 ± 45.0 nm/Pa for males and 108 ± 61.2 nm/Pa for females (Table A1.2, Figure A1.4b).

Table A1.2. Vibration characteristics of tympanal membrane in males and females. Significant difference between sexes was determined using unpaired Student's t-test for unequal variances ($p<0.01$ taken as significant).

Parameter	Intact		p-value	Ablated		p-value
	Male (n=7)	Female (n=7)		Male (n=7)	Female (n=7)	
Mean resonance frequency (kHz) (± s.d.)	8.03 ± 1.11	7.54 ± 1.34	0.237	8.13 ± 0.94	8.02 ± 2.09	0.451
Mean displacement at resonance frequency (nm/Pa) (± s.d.)	162 ± 98.1	149 ± 88.2	0.384	111 ± 40.3	85.2 ± 54.6	0.168
Mean maximum displacement (nm/Pa) (± s.d.)	211 ± 105	196 ± 100	0.388	136 ± 45.0	108 ± 61.2	0.175
Frequency at maximum displacement (kHz)	4.68 ± 3.0	4.09 ± 3.28	0.365	7.18 ± 1.63	8.09 ± 4.62	0.319

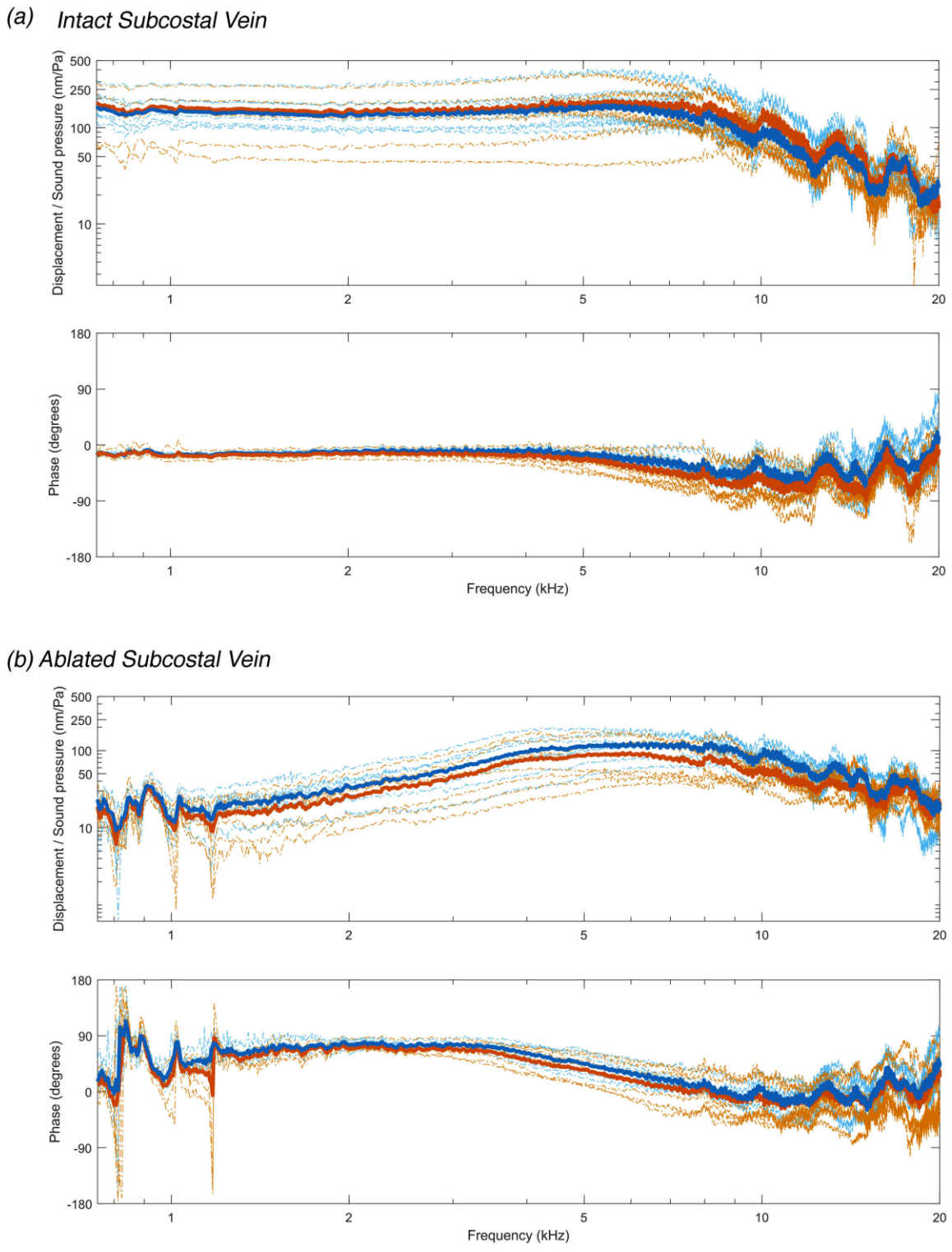


Figure A1.4. Male and female membrane displacement and phase response for intact (a) and ablated (b) conditions (light blue: 7 males; solid dark blue line: male mean; light orange: 7 females; solid red-orange line: female mean). Coherence was kept over 0.8 for both conditions.

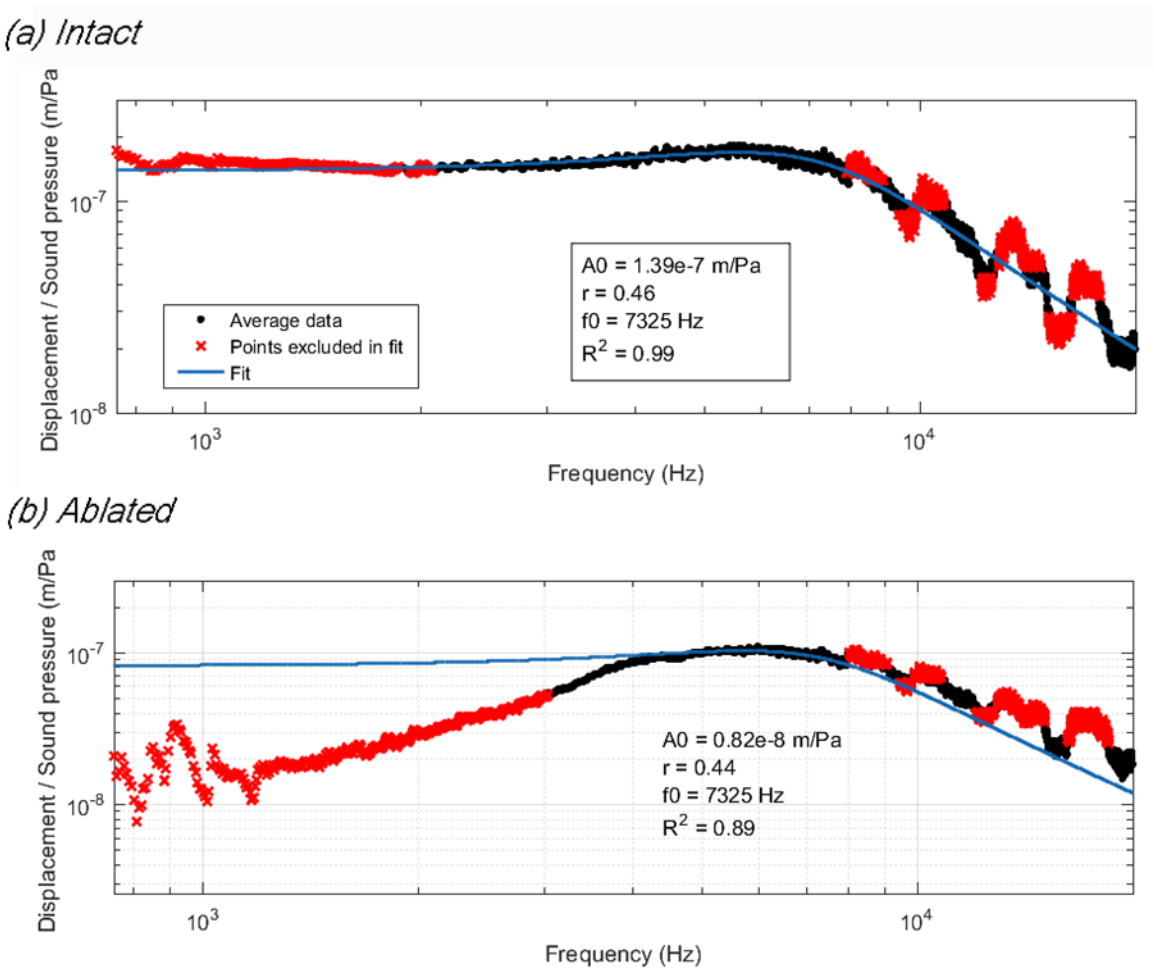


Figure A1.5. Lines of fit to the averaged data across all animals (corresponding to data presented in Figure 2.2. of main text Chapter 2) show that the harmonic oscillatory model is appropriate to describe the system.

Movie A1.1. Tympanal membrane mechanics. Tympanal displacement of animal 8 at its resonance frequency, 5.756 kHz, under intact and ablated conditions. Note the difference in displacement scales between conditions. (URL will be included online in published paper).

Appendix 2: Comparative study supplementary material

A2.1. Comparative specimens and measurements

Table A2.1. List of species examined showing origin of specimen, number of males and females tested, and the date tested. All specimens measured by Penghui Sun, all specimens from McGuire Center sent from Akito Y. Kawahara.

Subfamily	**Tribe**	**Species**	**Origin(s)**	**Number tested (M, F)**
Satyrinae	Satyrini	*Cercyonis pegala*	Yack lab, Carleton University Sharbot Lake, ON (live, Aug 2017) West End Lane, ON (live, Aug 2017) Perth, ON (live, Aug 2017)	15M, 15F
Satyrinae	Satyrini	*Coenonympha tullia*	Canadian National Collections North Cumberland, ON (Aug 20, ’98) Macoun Marsh, ON (live, June 2017)	3M, 2F
Satyrinae	Satyrini	*Coenonympha nipisquit*	Bathurst, NB, sent to Yack lab, Carleton University	1M
Satyrinae	Satyrini	*Enodia anthedon*	Yack lab, Carleton University Manion Corners, ON (live)	3M, 1F
Satyrinae	Satyrini	*Megisto cymela*	Guelph, Ontario (June 16, ’13) Yack lab, Carleton University (Sent to Yack lab, Carleton University)	1M, 3F
Satyrinae	Satyrini	*Satyrodes appalachia*	Manion Corners, ON (live) Thames Bush, Prescott, ON (Sept 7, ’86, from R. Layberry) (Sent to Yack lab, Carleton University)	2M
Satyrinae	Satyrini	*Satyrodes eurydice*	Yack lab, Carleton University Manion Corners, ON (live)	2M
Satyrinae	Satyrini	*Erebia discoidalis*	68°08'00.0"N 123°28'00.0"W, NWT (Mar 5, ’12, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Erebia fasciata*	Daring Lake, NWT (Aug 5, ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Erebia mackinleyensis*	Palmer Study Area, Alasdair, PEI (July 26, ’10, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Erebia mancinus*	Jawbone Lake, NWT (2011, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Erebia youngi*	Mackenzie Mountains, NWT (July 23, ’11, from R. Layberry) Black Shale, NWT (July 23, ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	2M
Satyrinae	Satyrini	*Oeneis bore*	Squirrel Hills, Mackenzie Mountains, NWT (June 23, ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	1F
Satyrinae	Satyrini	*Oeneis chryxus*	Gun Range, Norman Wells, NWT (June 19 ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Oeneis jutta*	Jawbone Lake, NWT (June 26, ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Oeneis polixenes*	Mackenzie Mountains, NWT (July 7, ’11, from R. Layberry) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Satyrini	*Hermeuptychia sosybius*	Florida, USA (Sept 9, ’98)	1M

			(Sent to Yack lab, Carleton University)	
Satyrinae	Satyrini	*Lethe diana*	Enzam, Japan (All sent from McGuire Center, University of Florida)	2M
Satyrinae	Satyrini	*Lethe sicelis*	Fujinomiya, Japan (All sent from McGuire Center, University of Florida)	3M
Satyrinae	Satyrini	*Lopinga achine*	Enzam, Japan (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Satyrini	*Neope niphonica*	Mt. Asama, Japan (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Satyrini	*Mycalesis francisca*	Unknown origin, from McGuire Center, University of Florida	1M
Satyrinae	Satyrini	*Minois dryas*	Unknown origin, from McGuire Center, University of Florida x2	2M
Satyrinae	Satyrini	*Ypthima albida*	Entebbe, Uganda (Nov 7, ’71) (Nov 17, ’71) (All sent from McGuire Center, University of Florida)	1M, 1F
Satyrinae	Satyrini	*Ypthima baldus*	Fujinomiya, Japan (All sent from McGuire Center, University of Florida)	2M, 1F
Satyrinae	Satyrini	*Bicyclus aurivilli*	Entebbe, Uganda (Nov 19, ’68) Bamboo Forest, Kigezi, South Africa (April 20, ’68) x2 (All sent from McGuire Center, University of Florida)	2M
Satyrinae	Satyrini	*Bicyclus denina*	Entebbe, Uganda (Mar 10, ’68) (Nov 19, ’68) Nombasa, Kenya (Dec 7, ’67) (All sent from McGuire Center, University of Florida)	1M, 1F
Satyrinae	Satyrini	*Bicyclus funebris*	Entebbe, Uganda (Nov 24, ’68) (All sent from McGuire Center, University of Florida)	1M, 1F
Satyrinae	Satyrini	*Bicyclus jeffreyi*	Entebbe, Uganda (Nov 19, ’68) (All sent from McGuire Center, University of Florida)	1F
Satyrinae	Satyrini	*Bicyclus mandanes*	Entebbe, Uganda (Nov 6, ’67) (Nov 8, ’68) (All sent from McGuire Center, University of Florida)	1M, 1F
Satyrinae	Satyrini	*Bicyclus sebetus*	Entebbe, Uganda (July 15, ’67) (All sent from McGuire Center, University of Florida)	1F
Satyrinae	Satyrini	*Heteropsis perspicua*	Umdoni Park, Pennington, South Africa (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Satyrini	*Pseudonympha trimenii*	Cathcart, South Africa (Oct 9, ’64) (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Satyrini	*Ypthimomorpha itonia*	Entebbe, South Africa (Nov 8, ’67) (All sent from McGuire Center, University of Florida)	1F
Satyrinae	Satyrini	*Ninguta schrenkii*	Akabira, Hokkaido, Japan (Aug 3, ’57) (Aug 10, ’57) (All sent from McGuire Center, University of Florida)	2M
Satyrinae	Satyrini	*Cassionympha cassius*	Buffalo Pass, East London Cape (Feb 6, ’74) Anabele forest, Stutterheim, South Africa (Feb 11, ’74) (All sent from McGuire Center, University of Florida)	2M
Satyrinae	Satyrini	*Stygionympha irrorata*	Edenburg, Sandton, South Africa (Feb 12, ’74) (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Morphini	*Morpho amathonte*	Unknown origin, sent to Yack lab, Carleton University	1M
Satyrinae	Morphini	*Morpho microthalmus*	London Pupae Supplies, sent to Yack lab, Carleton University	1F
Satyrinae	Morphini	*Morpho peleides*	London Pupae Supplies (Oxford, UK: P-2007-01460) (Sent to Yack lab, Carleton University)	1M, 2F
Satyrinae	Brassolini	*Caligo eurilochus*	London Pupae Supplies (Horspath, Oxfordshire, UK; P-2007-01460) (Sent to Yack lab, Carleton University)	1M, 1F
Satyrinae	Brassolini	*Caligo illioneus*	Santender, Columbia (sent to Yack lab, Carleton University)	1M
Satyrinae	Brassolini	*Caligo memnon*	London Pupae Supplies, sent to Yack lab, Carleton University)	2M, 1F

Satyrinae	Elymiini	*Elymniosis bammakoo*	R. Layberry (Aug 4, ’11) (Sent to Yack lab, Carleton University)	1M
Satyrinae	Elymiini	*Elymnias kanekei*	Unknown origin, sent to Yack lab, Carleton University	1M
Satyrinae	Haeterini	*Pierella astyoche*	Unknown origin, sent to Yack lab, Carleton University	1M
Satyrinae	Melanitini	*Melanitis leda*	Entebbe, South Africa (Oct 25, ’72) (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Melanitini	*Melanitis phedima*	Fujinomiya, Japan (July 22, ’17) (All sent from McGuire Center, University of Florida)	1M
Satyrinae	Melanitini	*Gnophodes betsimena*	Entebbe, South Africa (Oct 29, ’71) (All sent from McGuire Center, University of Florida)	1F
Satyrinae	Dirini	*Paralethe dendrophilus*	Stutterheim, Eastern Cape, South Africa (Feb 14, ’71) Evelyn Valley, South Africa (Feb 11, ’71) (All sent from McGuire Center, University of Florida)	1M, 1F
Satyrinae	Amathusiini	*Amathusia phidippus*	Unknown origin, sent to Yack lab, Carleton University	1M
Satyrinae	Zetherini	*Zethera musa*	Insectes Mondieaux from J. King, sent to Yack lab, Carleton University	1M
Charaxinae	Charaxini	*Charaxes howarthi*	Unknown origin, sent to Yack lab, Carleton University	1M
Charaxinae	Charaxini	*Charaxes subornatus*	Unknown origin, sent to Yack lab, Carleton University	1M
Charaxinae	Charaxini	*Consul fabius*	C. Preston (captured Aug 20 ’12) x2 (sent to Yack lab, Carleton University)	2F
Charaxinae	Charaxini	*Polyura athamas*	Unknown origin, from Yack lab, Carleton University	1U (no abdomen to sex)
Charaxinae	Charaxini	*Archaeprepon demophon*	London Pupae Supplies, sent to Yack lab, Carleton University	1M
Charaxinae	Prothonini	*Prothoe franck*	Siberut, Indonesia, to Insects Mondiaux (Feb 22 ’08, from J.King) (Sent to Yack lab, Carleton University)	1M
Charaxinae	Pallini	*Palla decius*	Insectes Mondiaux (Mar 8, ’08, from J. King) (sent to Yack lab, Carleton University)	1M
Heliconiinae	Acraeini	*Acraea viola*	Thailand, sent to Yack lab, Carleton University	1M
Heliconiinae	Acraeini	*Cethosia cyane*	Unknown origin, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Dione juno*	Maracay, Aragua, Venezula (Sept, ’88) sent to Peru, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Heliconius charitonia*	Unknown origin captured live (Oct 19, ’11), from L. McMillan, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Heliconius erato*	Unknown origin captured live (Oct 18, ’11), from L. McMillan, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Heliconius hecale*	Unknown origin captured live (Oct 18, ’11), from L. McMillan, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Heliconius melpomene*	Unknown origin captured live (Oct 19, ’11), from L. McMillan, sent to Yack lab, Carleton University	1M
Heliconiinae	Heliconiini	*Dryadula phaetusa*	Unknown origin, sent to Yack lab, Carleton University	1F
Heliconiinae	Heliconiini	*Dryas iullia*	Unknown origin, sent to Yack lab, Carleton University	1F
Heliconiinae	Argyniini	*Argynnis aphrodite*	Carleton Lands, Ottawa, ON (June 26, ’11, from L. Hall) (Sent to Yack lab, Carleton University)	1M
Heliconiinae	Vagrantini	*Cirrochroa regina*	Nabire, Indonesia, to Insects Mondiaux (Dec 2003, from J.King) (Sent to Yack lab, Carleton University)	1M
Danaiinae	Danaini	*Danaus chrysippus*	Unknown origin, sent to Yack lab, Carleton University	1M
Danaiinae	Danaini	*Danaus cleophile*	Unknown origin, sent to Yack lab, Carleton University	1M
Danaiinae	Danaini	*Danaus petilia*	Unknown origin, sent to Yack lab, Carleton University	1M
Danaiinae	Danaini	*Danaus plexippus*	Unknown origin (Aug 5, ’11) (Sent to Yack lab, Carleton University)	1M
Danaiinae	Danaini	*Tirumala limniace*	Unknown origin, sent to Yack lab, Carleton University	1F

Danaiinae	Danaini	*Idea leuconoe*	Unknown origin captured live from L. McMillan, sent to Yack lab, Carleton University	1M, 1F
Nymphalinae	Junonini	*Hypolimnas bolima*	London Pupae Supplies, sent to Yack lab, Carleton University	1M, 1F
Biblidinae	Ageroniini	*Panacea prola*	Unknown origin, from Yack lab, Carleton University	1M
Apaturinae	NA	*Doxocopa pavon*	Unknown origin from Yack lab, Carleton University	1F

Table A2.2. Example of practice species measurements (right-side).

Species/ID	FW Sc width (µm)				HW Sc width (µm)				FW Cu width (µm)				HW Cu width (µm)			
	1	2	3	x̄	1	2	3	x̄	1	2	3	x̄	1	2	3	x̄
Doxocopa pavon 060117 YL F	290.0	360.7	359.91	337.2	178.43	177.45	170.17	175.35	259.68	282.01	316.71	286.13	166.82	180.0	163.36	170.06

FW An width (µm)				HW An width (µm)				VO length (µm)				VO width (µm)			
1	2	3	x̄	1	2	3	x̄	1	2	3	x̄	1	2	3	x̄
317.71	328.88	337.91	328.17	127.03	163.36	136.63	142.34	1003.24	980.76	982.69	988.9	565.04	596.25	606.51	589.27

VO surface area (µm²)			
1	2	3	x̄
439302.83	496223.78	464923.21	466816.61

A2.2. Using ratio method 2: Reference vein ratio for vein and ear categories

When using ratio method 2 to quantify the vein, proportions of vein and ear categories are slightly different, but generally agrees between ratio methods (Figure A2.1, A2.2). When using this ratio, the species with the lowest ratio that still had a "large" rating from ratio method 1 is *Neope niphonica* (3.7007). However, this creates different species to be considered as thick, as they have ratios higher than 3.7007, including *Danaus plexippus, Zethera musa, Heliconius erato, Heliconius melpomene,* and *Danaus chrysippus*. *Panacea prola* remains as "thick" between both ratio methods, but the other 2 species (*Danaus cleophile, Archaeprepon demophon)* that were classified as thick using ratio method 1 are now classified as "small." For ratio method 2, all species categorized as large also have well-developed ears.

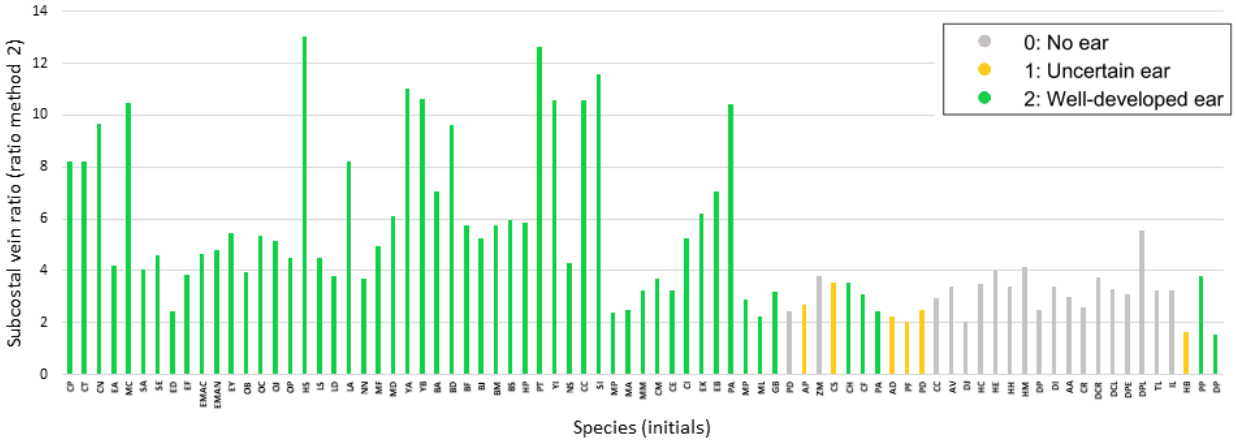


Figure A2.1. Vein ratios types categorized with ear types, using ratio method 1 for all species sampled.

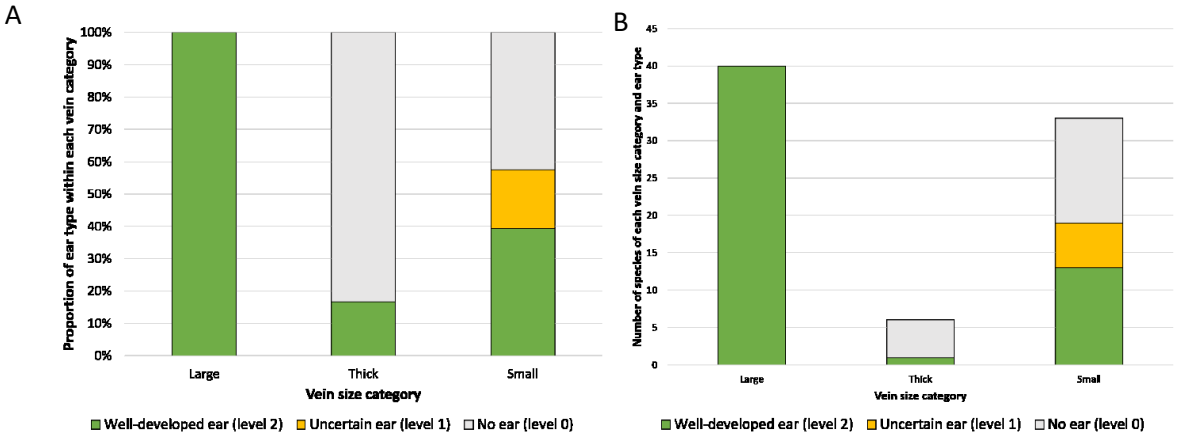


Figure A2.2. Ear types and vein sizes of the species sampled using ratio method 2. (A) Proportion of vein sizes and ear types for all species sampled. (B) Number of species for each vein size and ear type.

A2.3. Sexual dimorphism

C. pegala: Body size using PC1 demonstrated no significant difference between males and females. Thus, t-tests with unequal variances could be performed to test sexual dimorphism in vein ratios. Neither vein ratios exhibited sexual dimorphism (Figure A2.3, Table A2.3). There was variation for vein ratio 2, ranging from 6.81-10.09.

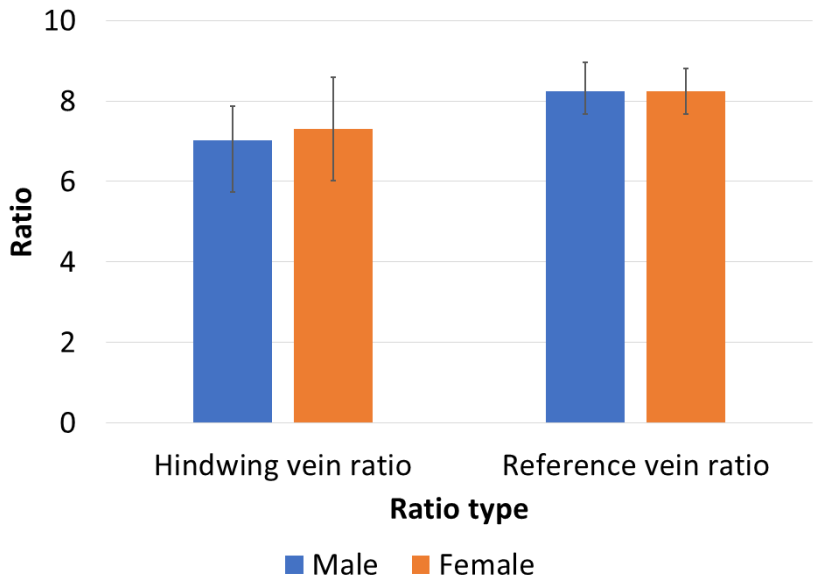
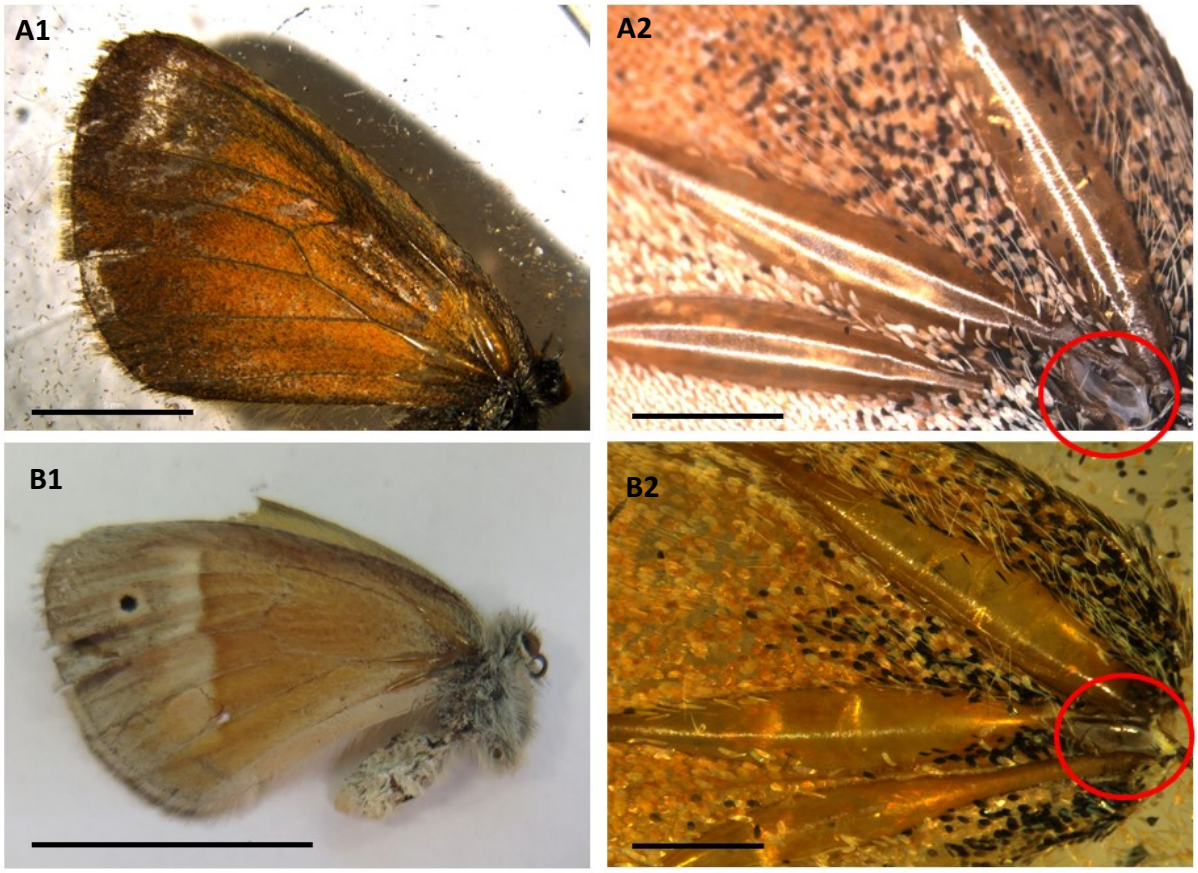


Figure A2.3. Subcostal vein ratios using both ratios methods, for both sexes in *C. pegala.*

Table A2.3. Sexual dimorphism in body size using PC1 was tested using unpaired t-test with unequal variances, $p<0.05$ taken as significant.[1] Sexual dimorphism in *C. pegala* for vein ratio 1 and 2, tested using unpaired t-test, with unequal variances, $p<0.05$ taken as significant.[2]

Ratio 1 (Hindwing vein)			**Ratio 2 (Reference vein)**			**Body size: PC1**		
Male	Female	p-value[2]	Male	Female	p-value[2]	Male	Female	p-value[1]
7.02 ± 0.85	7.30 ± 1.29	0.10	8.24 ± 0.73	8.24 ± 0.57	0.85	6.96E-14 ± 23.01	-4.69E-14 ± 39.24	1

C. tullia: Body size of *C. tullia* did not exhibit any signs of sexual dimorphism in body size, thus vein width and ratios between sexes could be directly compared via unpaired t-test with unequal variances (Figure 3.13, Table A2.4). Male vein width ranged from 651.52-766.02 µm, with FW:HW ratios ranging from 4.98-8.60. and FW:ref ratios ranging from 6.69-7.96. Female vein width ranged from 795.0-766.015 µm, with FW:HW ratios ranging from 5.30-5.72, and FW:ref ratios ranging from 7.19-9.08.



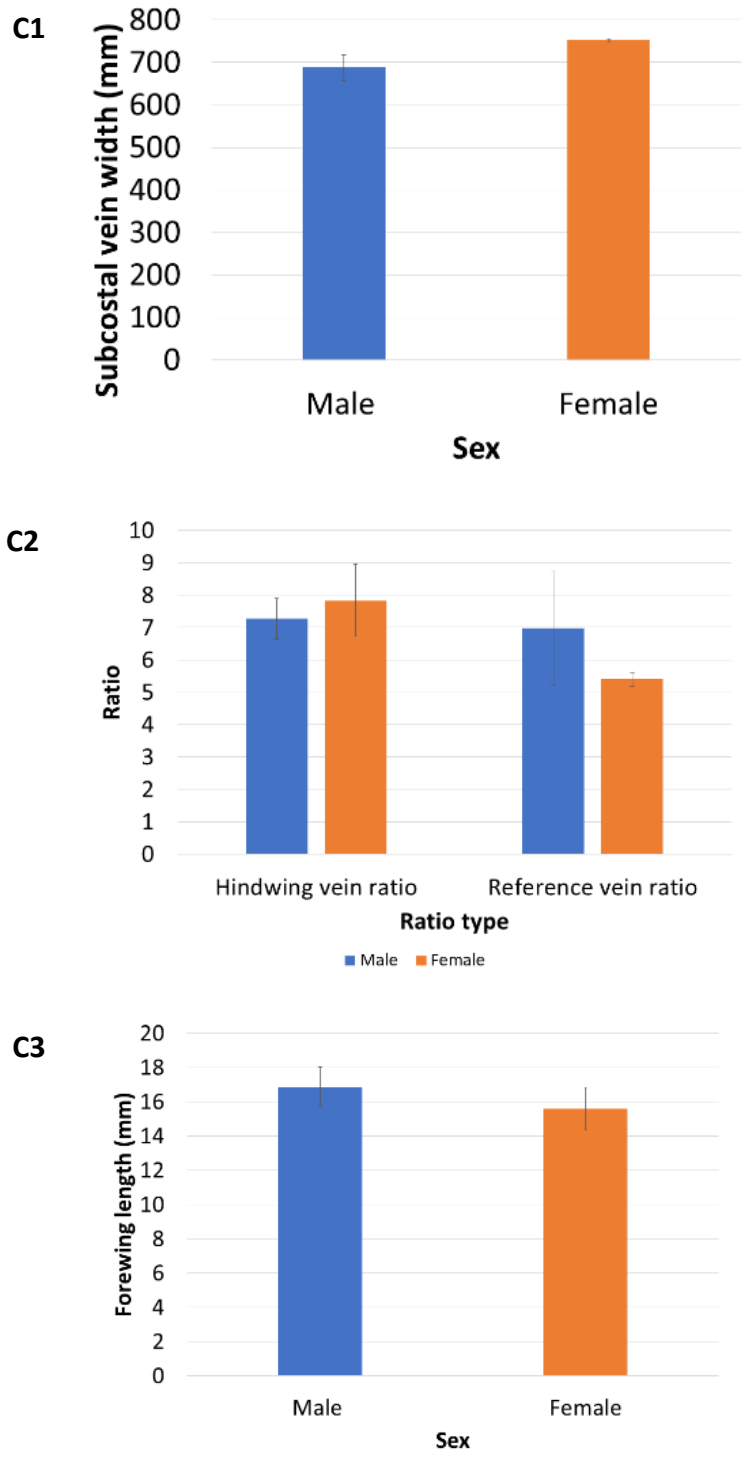


Figure A2.4. Male and female examples of morphology in *C. tullia*. (A1) Whole male on ventral side, hindwing detached, scale bar = 1 mm, (A2) Male forewing, circle denotes ear, scale bar = 2 mm, (B1) Whole female on ventral side, hindwing detached, scale bar = 1 mm, (B2) Female forewing, circle denotes ear, scale bar= 2 mm, (C1) Forewing length between sexes, with standard deviation bars, (C2) Subcostal vein ratio between sexes, with standard deviation bars, (C3) Subcostal vein width, between sexes, with standard deviation bars.

Table A2.4. Sexual dimorphism for *C. tullia.* Body size using PC1 was tested using unpaired t-test with unequal variances, $p<0.05$ taken as significant.[1] Sexual dimorphism in vein ratios, tested using unpaired t-test, with unequal variances, $p<0.05$ taken as significant.[2]

Subcostal vein width (mm)			**Ratio 1 (Hindwing vein)**			**Ratio 2 (Reference vein)**			**Body size: PC1**		
Male	Female	p-value[2]	Male	Female	p-value[2]	Male	Female	p-value[2]	Male	Female	p-value[1]
687.62 ± 29.69	752.74 ± 3.19	0.063	6.98 ± 1.74	5.40 ± 0.21	0.26	7.27 ± 0.63	7.83 ± 1.12	0.64	-8.3E-15 ± 5.94	9.77E-15 ± 13.96	1

A2.4. Relationship between body size and vein size, using ratio measure 2

The relationship between body size and vein ratio size when using ratio measure 2 generally shows the same trends as using ratio measure 1 (Figure A2.5, A2.6). The two-term exponential relationship between body size and vein size also generally agrees with using ratio measure 1 but is slightly stronger when using ratio 2 for most analyses (Table A2.5).

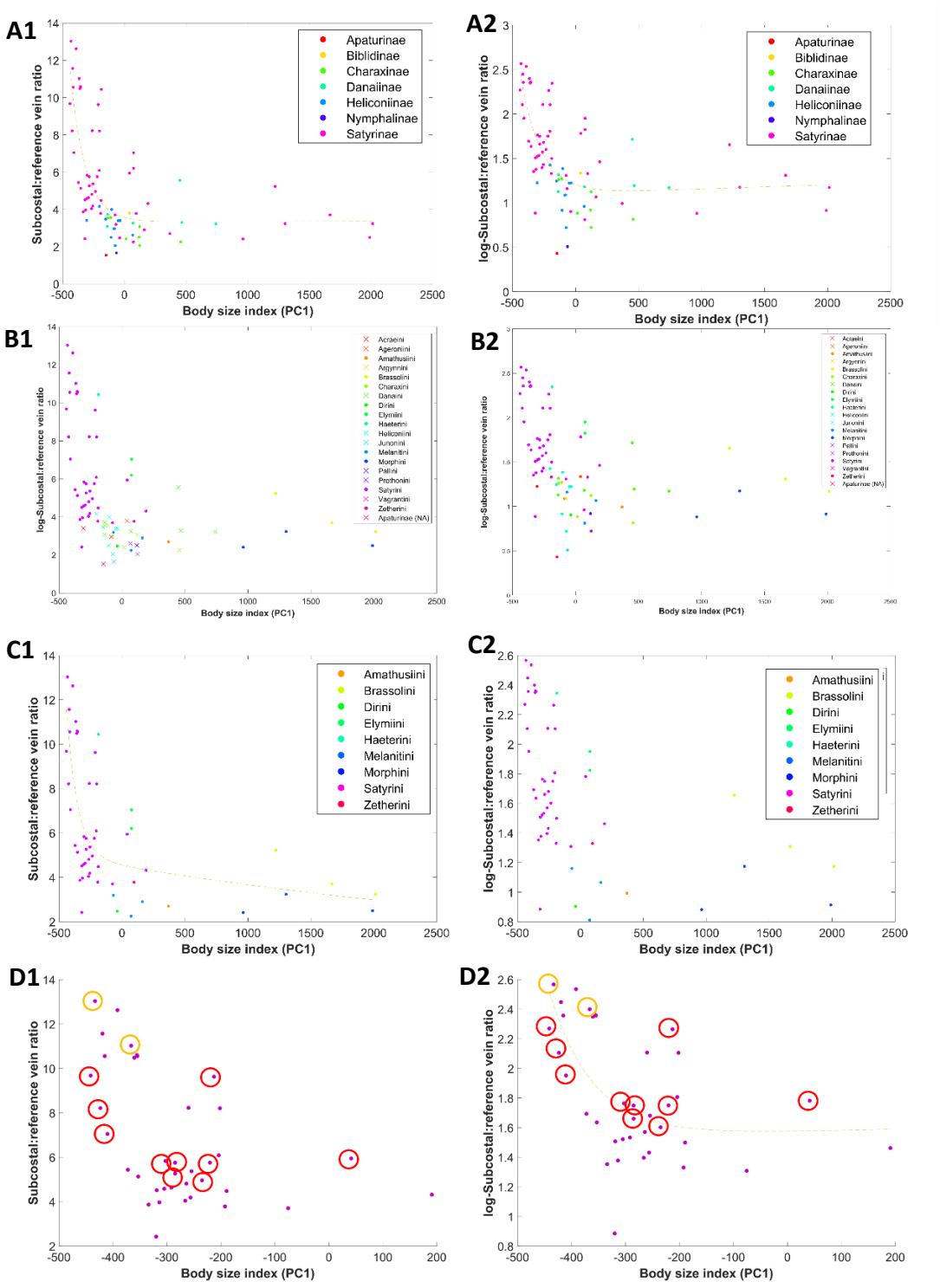


Figure A2.5. Relationship between body size (PC1) and subcostal forewing:hindwing vein ratio (ratio method 2). Left: Linear plots, right: Log-transformed plots. Dashed pale yellow line in A, C, D = 2-term exponential fit. (A) All species sampled, sorted by Subfamily. (B) All species sampled, sorted by Tribe. Tribes denoted by X: Tribes outside Satyrinae. (C) Satyrinae species sampled, sorted by Tribe. (D) Satyrini species sampled. Red circles: Species with enlarged Cubital and Anal veins as well, yellow circles: Species with enlarged Cubital vein as well (Table 3.1).

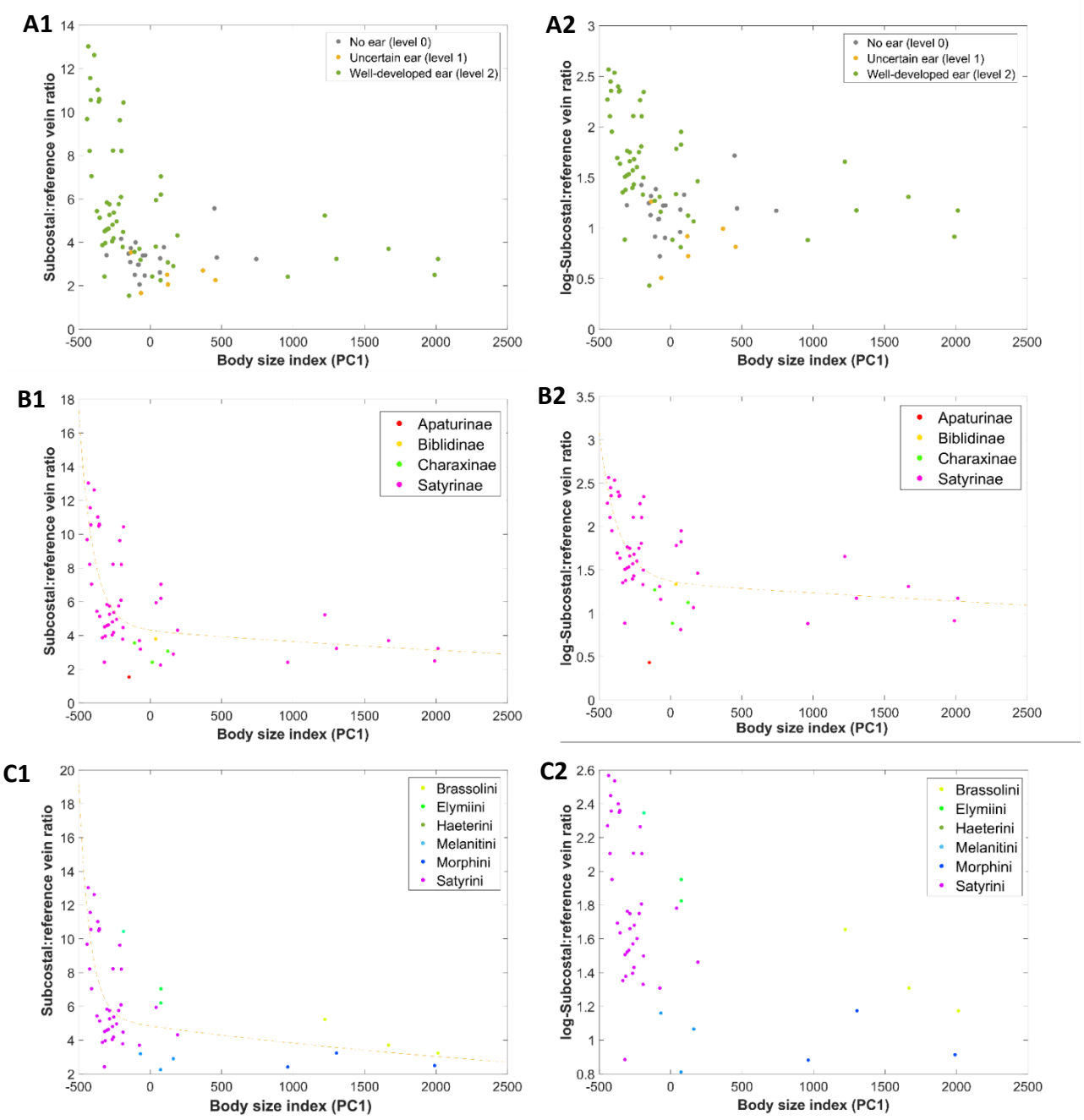


Figure A2.6. Relationship between body size (PC1) and vein size, using subcostal forewing:hindwing vein ratio (ratio method 2). Left: Linear plots, right: Log-transformed plots. (A) All species sampled, sorted by ear state, (B) Only eared species (level 2 ears), sorted by subfamily. Dashed pale yellow line = 2-term exponential fit. (C) Only Satyrinae species, with ears (level 2 ears), sorted by tribe. Dashed pale yellow line = 2-term exponential fit.

Table A2.5. Regression model fits for relationship between body size and vein ratio

	Linear regression fit R^2		1-term exponential regression fit R^2		2-term exponential regression fit R^2	
	Ratio measure 1	Ratio measure 2	Ratio measure 1	Ratio measure 2	Ratio measure 1	Ratio measure 2
All, linear	0.1562	0.1467	0.3536	0.3279	0.4682	0.5389
All, log-transformed	0.1782	0.147	0.2852	0.1994	0.4519	0.4883
Satyrinae only, linear	0.2196	0.201	0.314	0.2832	0.4077	0.4825
Satyrinae only, log-transformed	0.3277	0.2447	0.3828	0.2788	0.4546	0.4605
Satyrini only, linear	0.1307	0.2263	0.1924	0.3208	0.2900	0.4322
Satyrini only, log-transformed	0.09113	0.1972	0.1107	0.2313	0.2453	0.3823
Eared species only, linear	0.2022	0.1783	0.3070	0.2799	0.4059	0.4839
Eared species only, log-transformed	0.2973	0.1986	0.3524	0.2302	0.4387	0.4406
Satyrinae eared species only, linear	0.2216	0.2014	0.2864	0.2556	0.3892	0.4306
Satyrinae eared species only, log-transformed	0.3464	0.2532	0.3836	0.2775	0.4491	0.4469

Table A2.6. Effect of taxa levels on ratio, using ANCOVA to account for body size as covariate (p-values). Any specific groups that had specific significant effects are included. ($p<0.01$)

	Ratio measure 1	Log-transformed ratio measure 1	Ratio measure 2	Log-transformed ratio measure 2
All tribes	0.0138	<0.00001	0.0038	<0.00001
All subfamilies	0.0004 (Satyrinae: 0.0013)	<0.00001 (Satyrinae: <0.00001)	0.0006 (Satyrinae: 0.0002)	<0.00001 (Satyrinae: <0.00001)
Satyrinae only tribes	0.3101	0.0365	0.081	0.0066
All eared subfamilies	0.1661	0.0201	0.0841	0.004 (Satyrinae: 0.0014)
All eared Satyrinae tribes	0.3324	0.0898	0.0754	0.0114

Appendix 3: Photos of representative species sampled

A3.1. Satyrinae

Satyrini

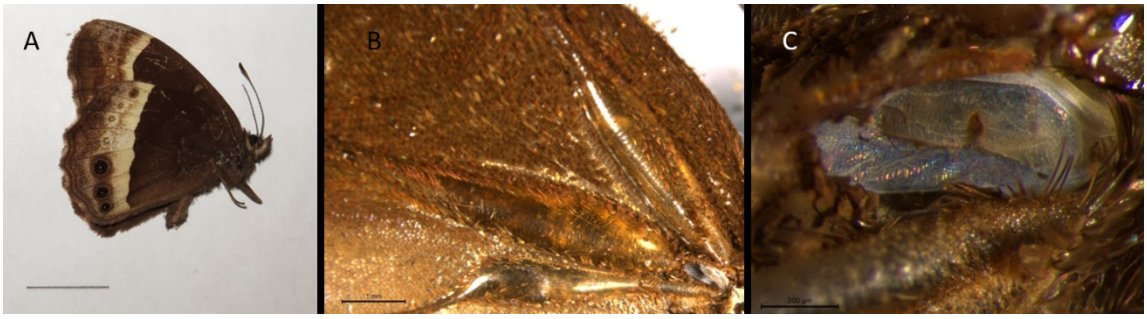


Figure A3.1. *Bicyclus aurivilli.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

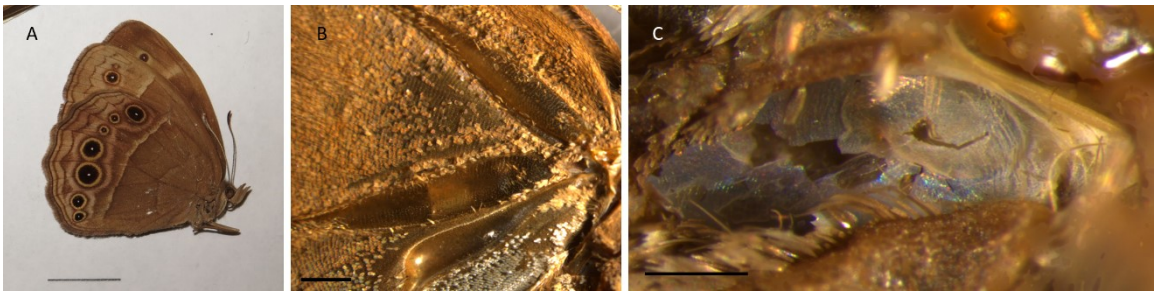


Figure A3.2. *Bicyclus denina.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

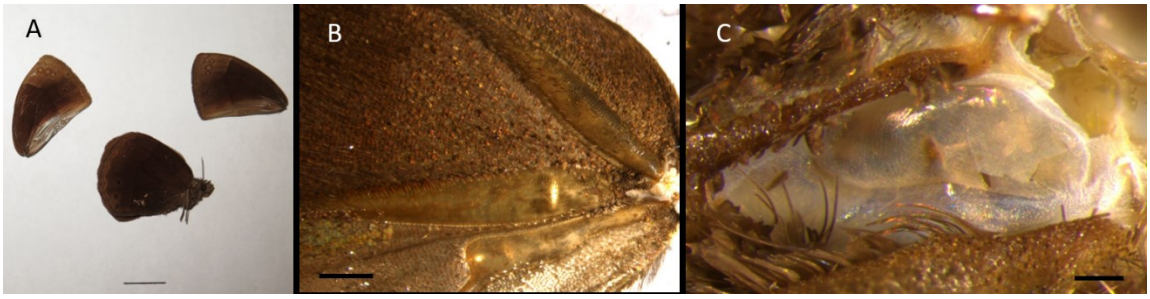


Figure A3.3. *Bicyclus funebris.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

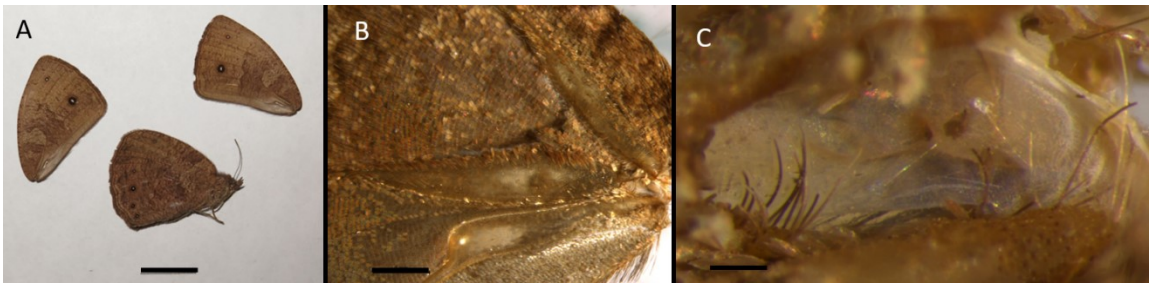


Figure A3.4. *Bicyclus jeffreyi.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

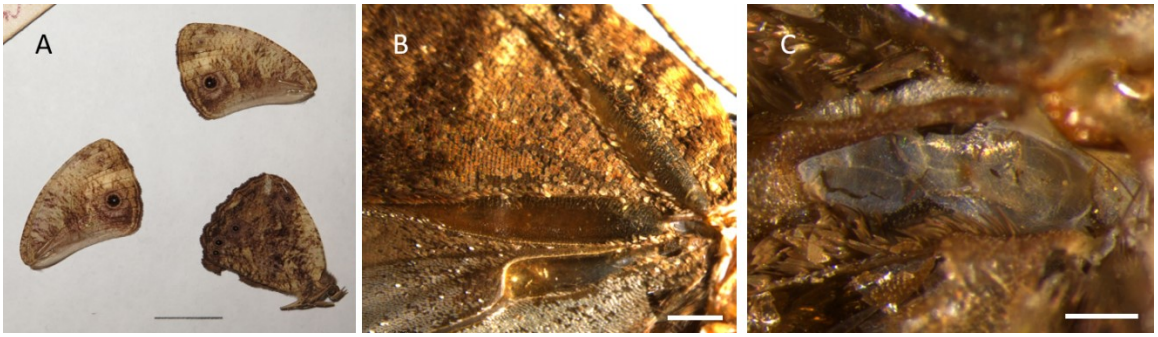


Figure A3.5. *Bicyclus mandanes.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

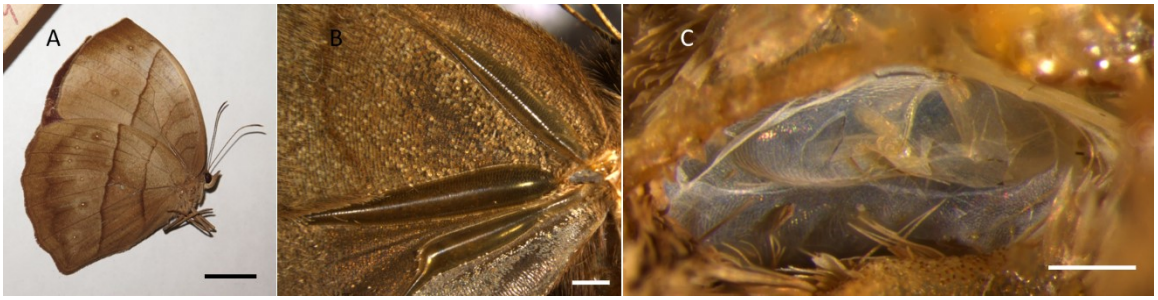


Figure A3.6. *Bicyclus sebetus.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

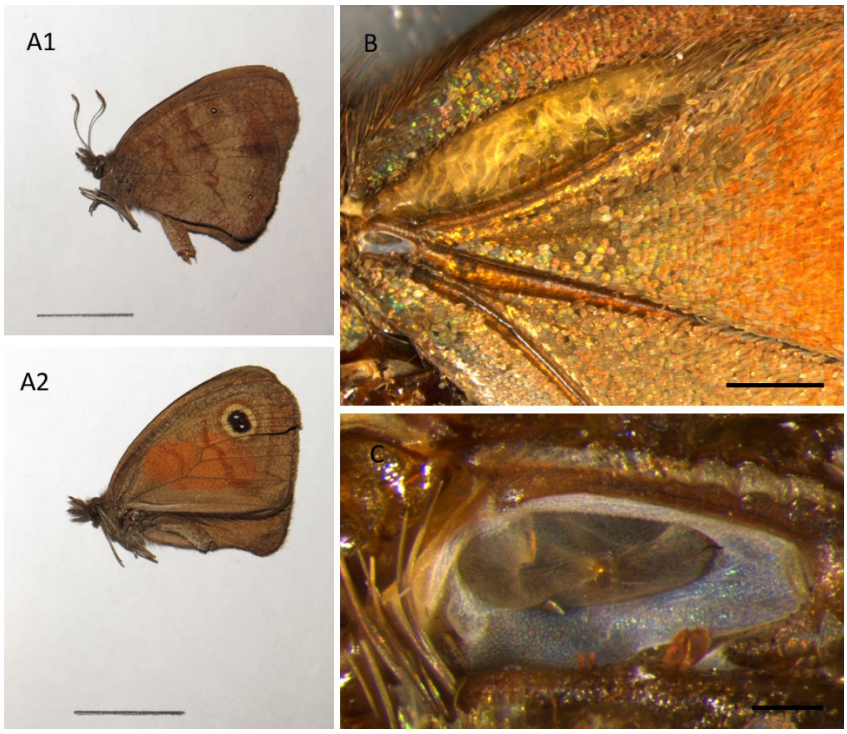


Figure A3.7. *Cassionympha cassius.* (A1) Whole butterfly on ventral side. Scale bar: 10 mm, (A2) Whole butterfly on ventral side, hindwing removed to show colour of forewing and eyespot. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

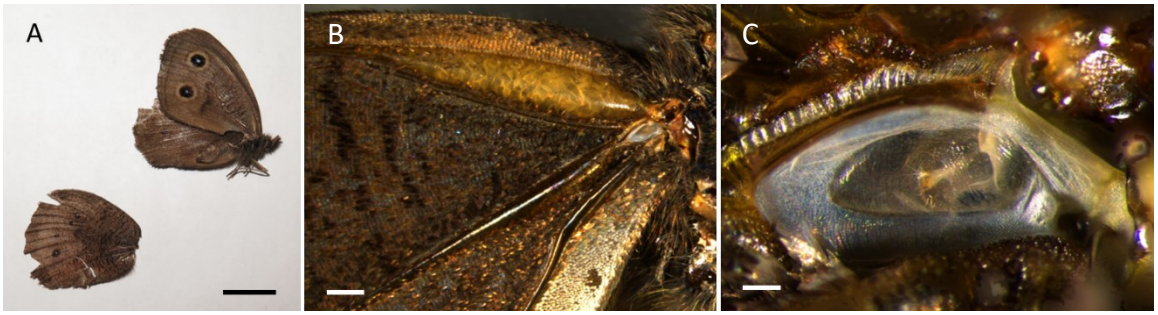


Figure A3.8. *Cercyonis pegala.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

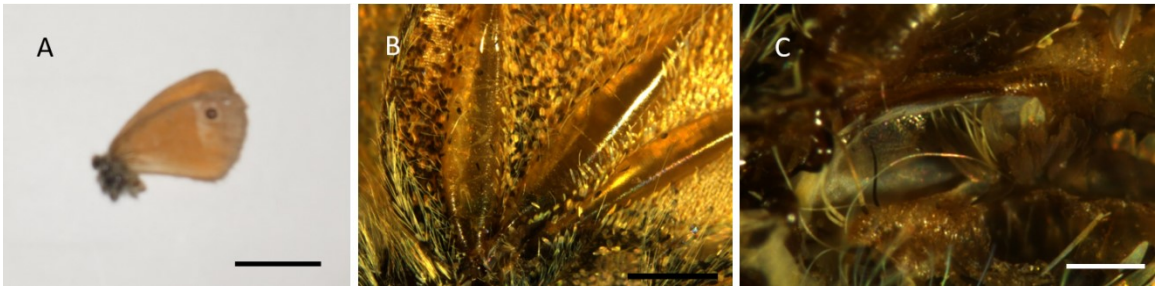


Figure A3.9. *Coenonympha nipisquit.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

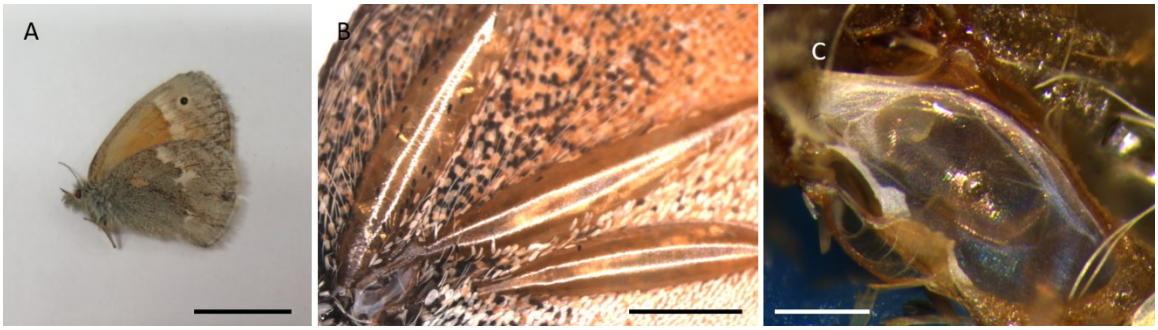


Figure A3.10. *Coenonympha tullia.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

Figure A3.11. *Enodia anthedon.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

Figure A3.12. *Erebia discoidalis.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

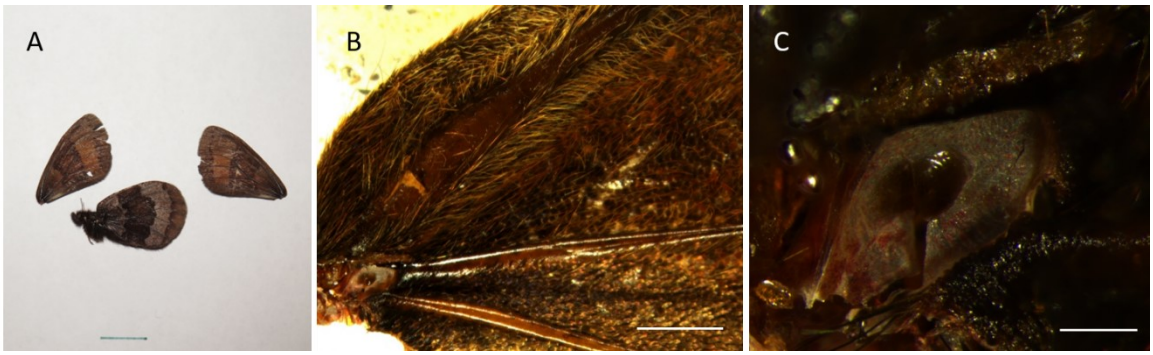


Figure A3.13. *Erebia fasciata.* (A) Whole butterfly on ventral side, hindwings removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

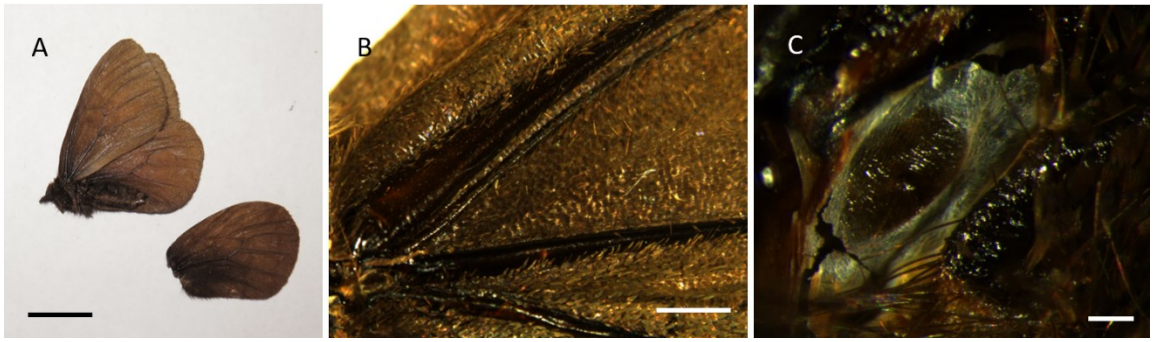


Figure A3.14. *Erebia mackinleyensis.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

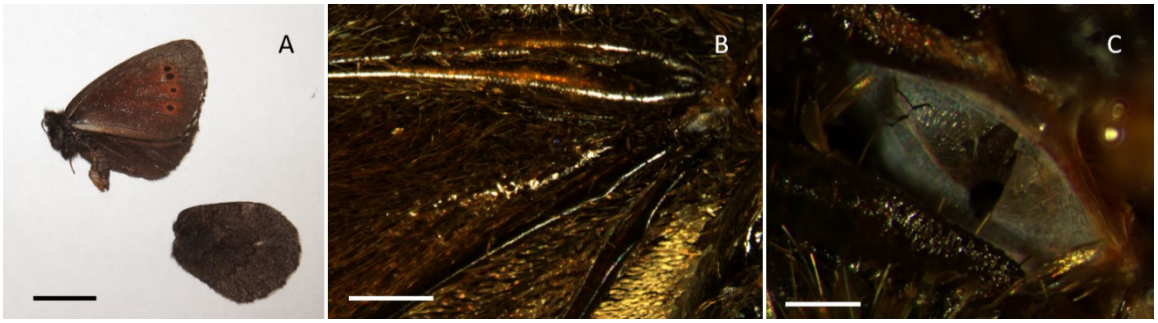


Figure A3.15. *Erebia mancinus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

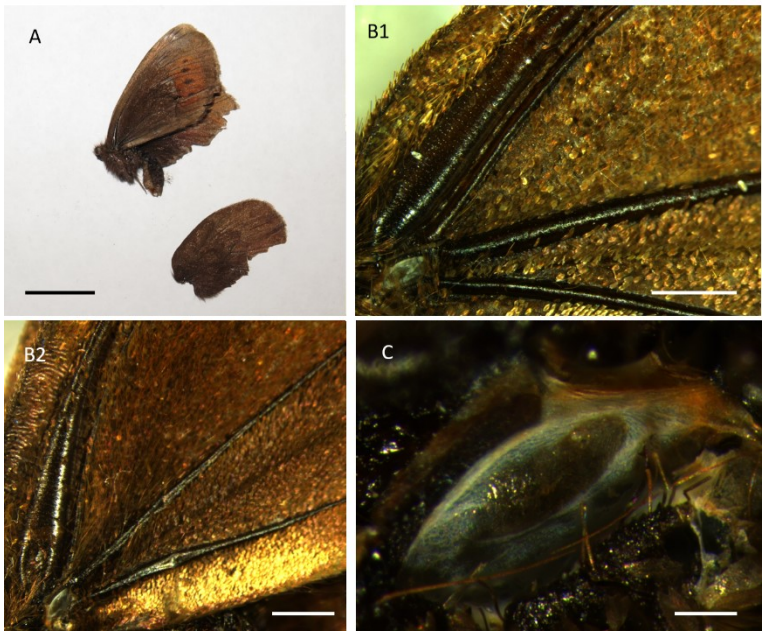


Figure A3.16. *Erebia youngi.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B1) Forewing on ventral side, scales removed. Scale bar: 1 mm, (B2) Second example of forewing on ventral side, scales removed, shown to show variation amongst specimens. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

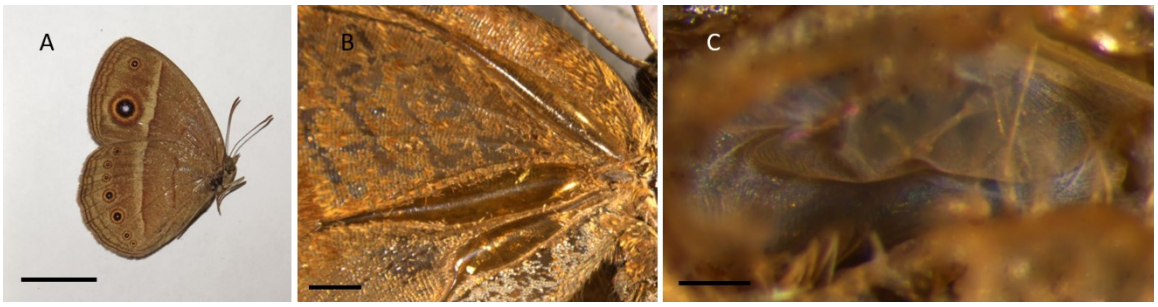


Figure A3.17. *Heteropsis perspicua.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

Figure A3.18. *Hermeuptychia sosybius.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

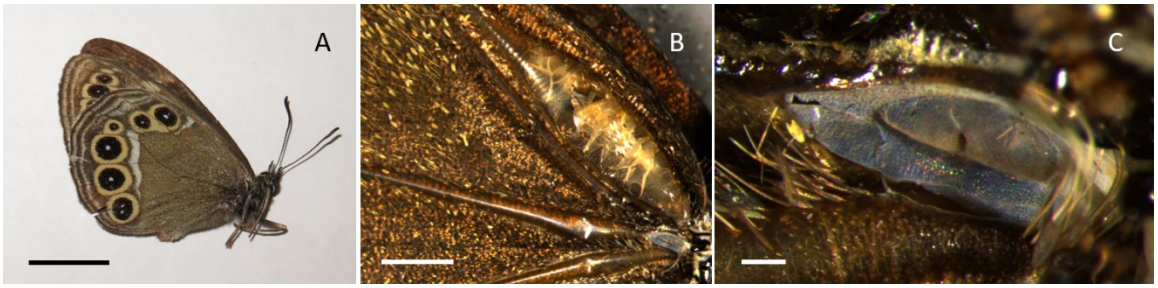


Figure A3.19. *Lopinga achine.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

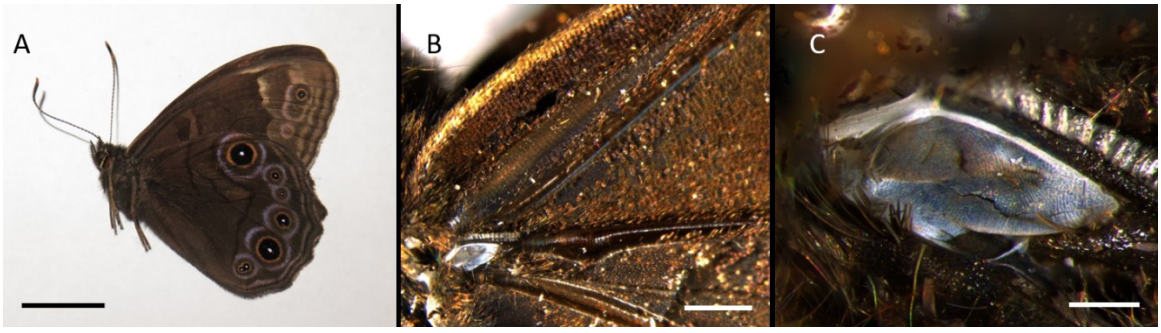


Figure A3.20. *Lethe diana.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

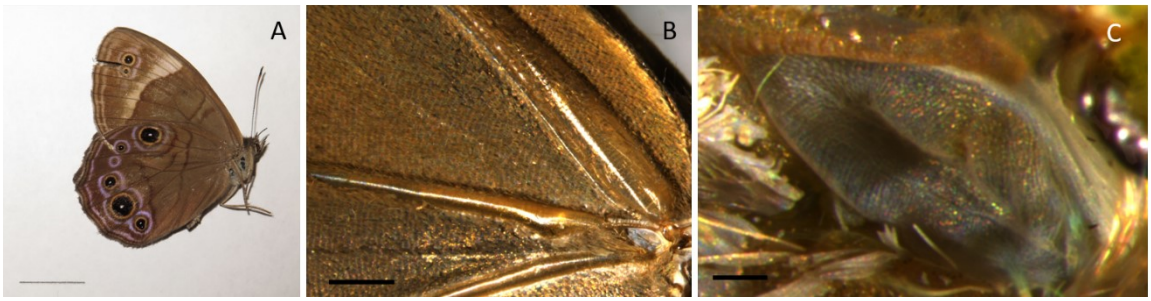


Figure A3.21. *Lethe sicelis.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

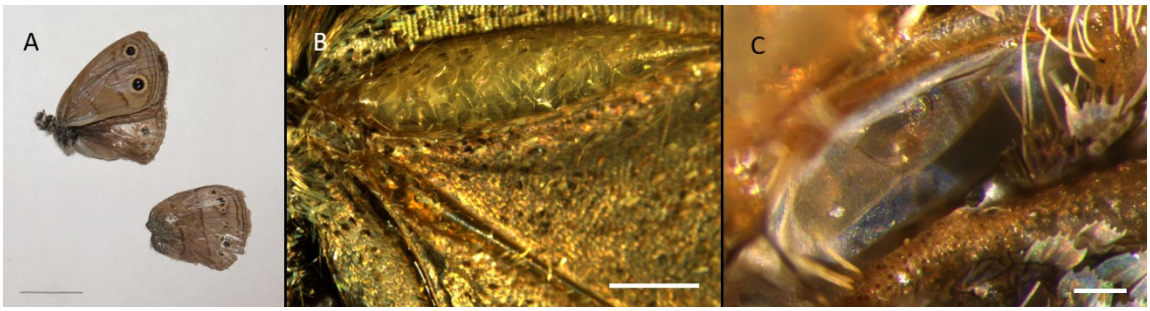


Figure A3.22. *Megisto cymela.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

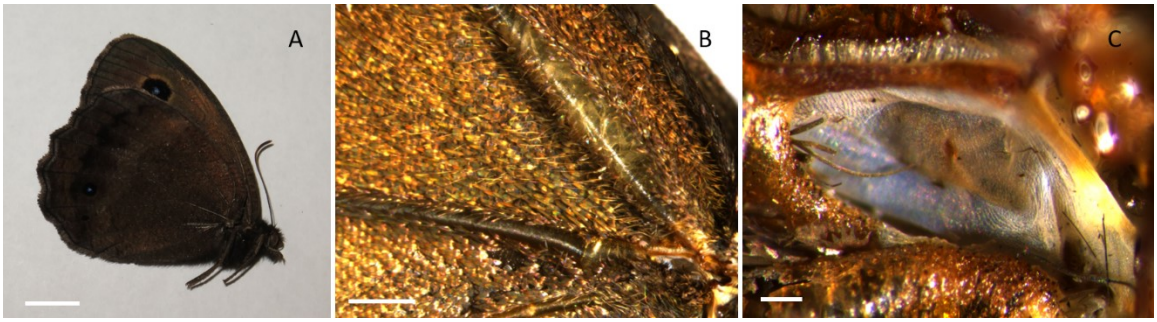


Figure A3.23. *Minois dryas.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

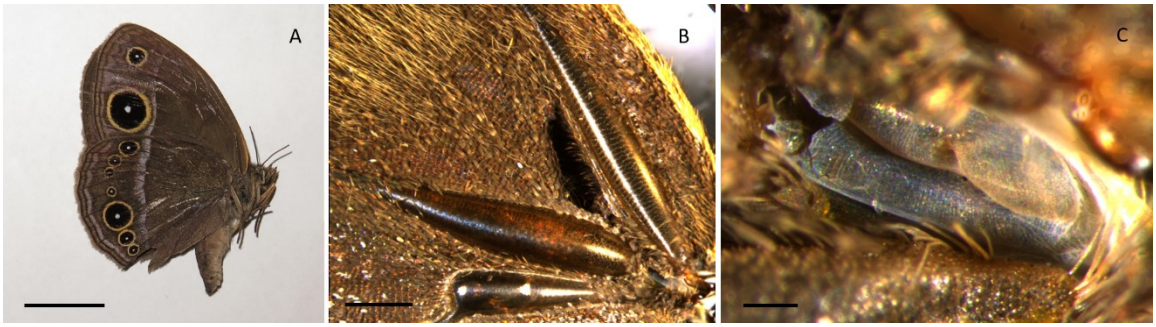


Figure A3.24. *Mycalesis francisca.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

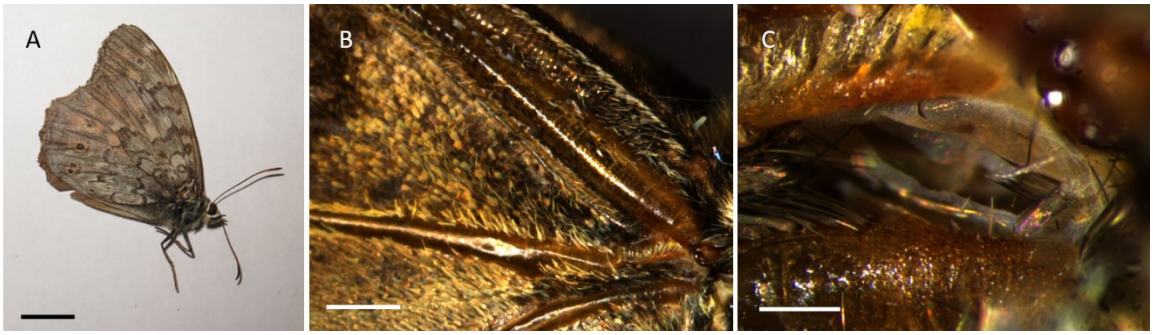


Figure A3.25. *Neope niphonica.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

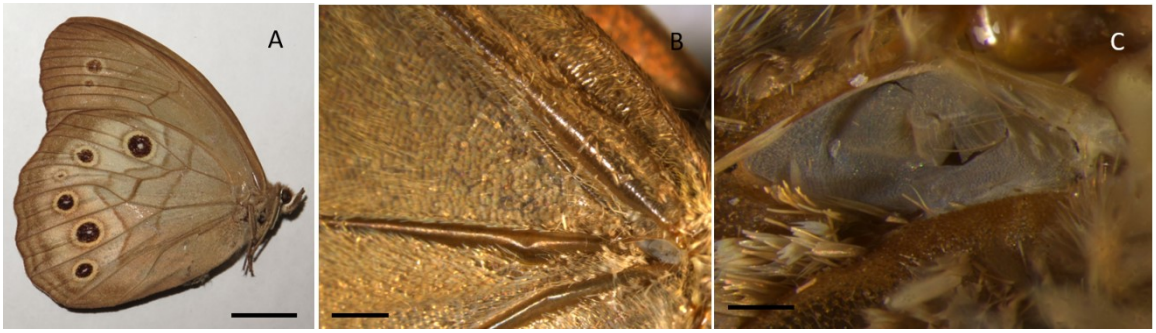


Figure A3.26. *Ninguta schrenkii.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

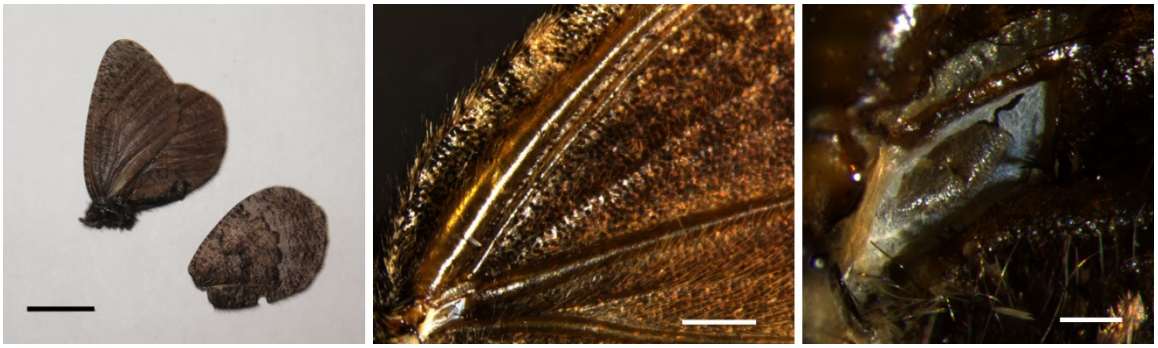


Figure A3.27. *Oeneis bore.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

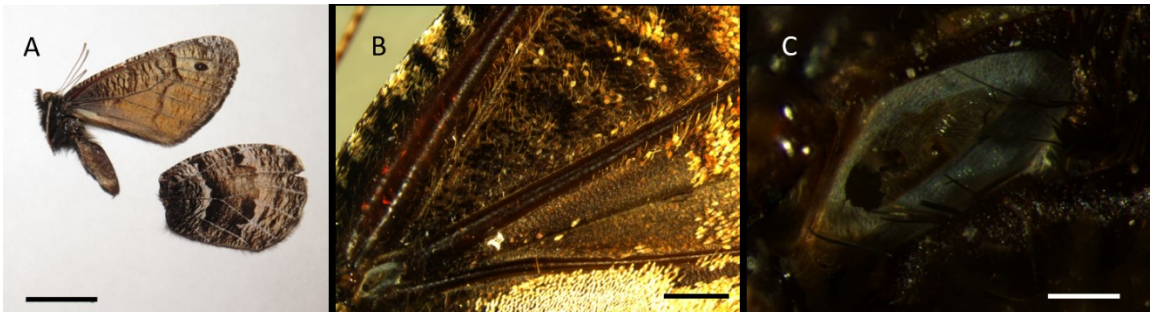


Figure A3.28. *Oeneis chryxus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

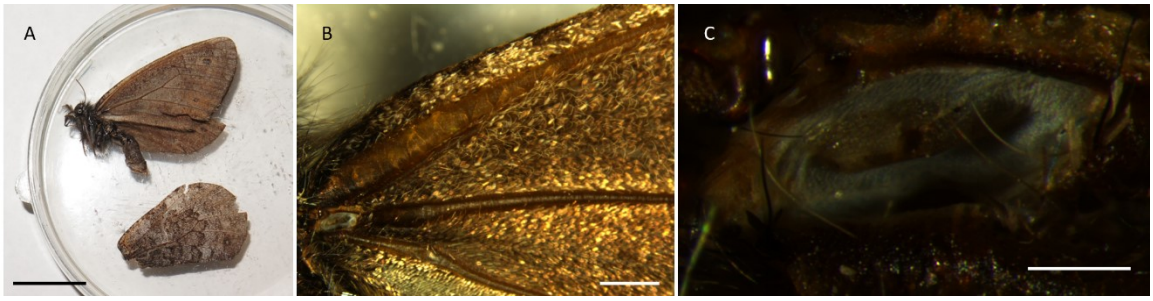


Figure A3.29. *Oeneis jutta.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

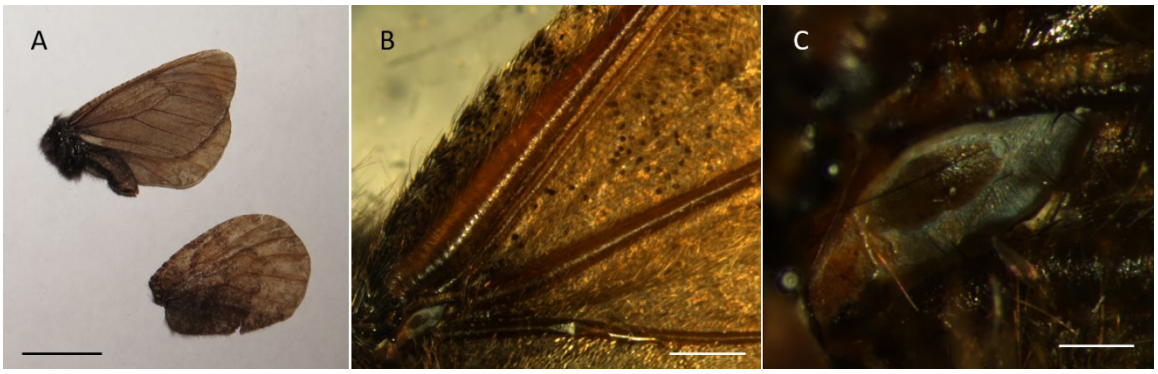


Figure A3.30. *Oeneis polixenes.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

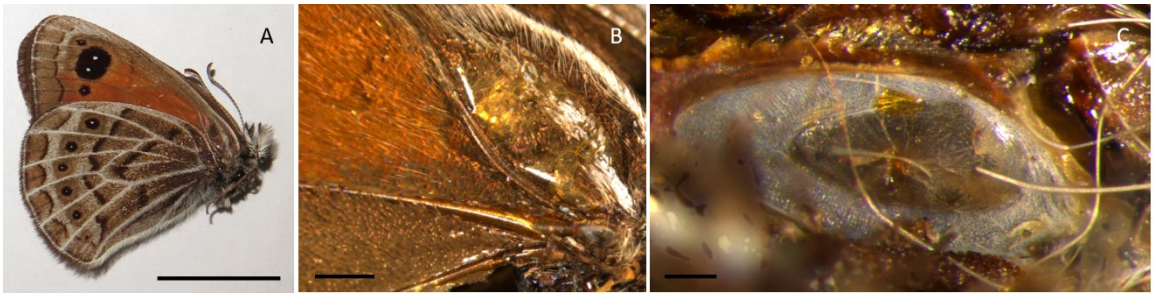


Figure A3.31. *Pseudonympha trimenii.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

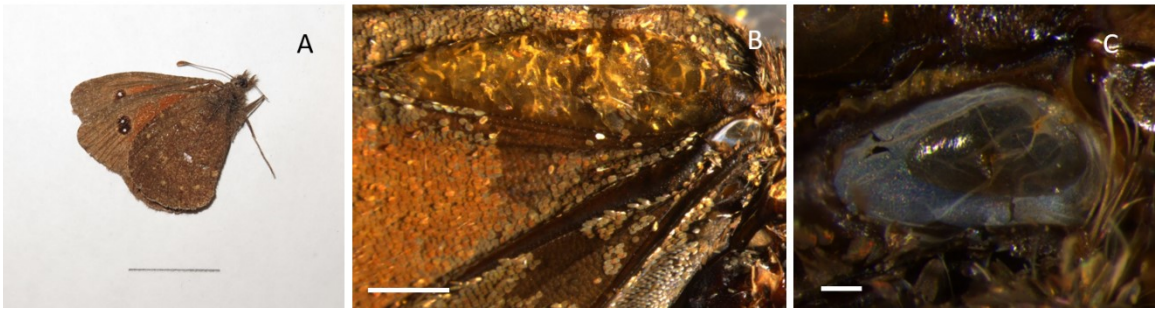


Figure A3.32. *Stygionympha irrorata.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

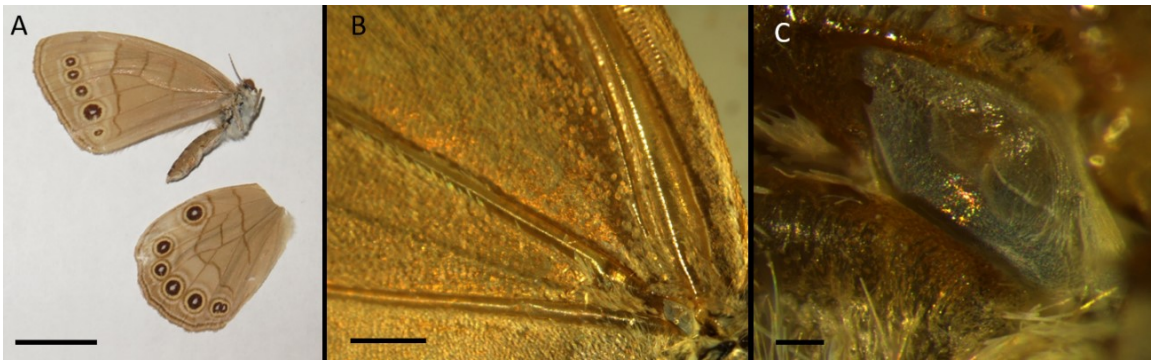


Figure A3.33. *Satyrodes appalachia.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

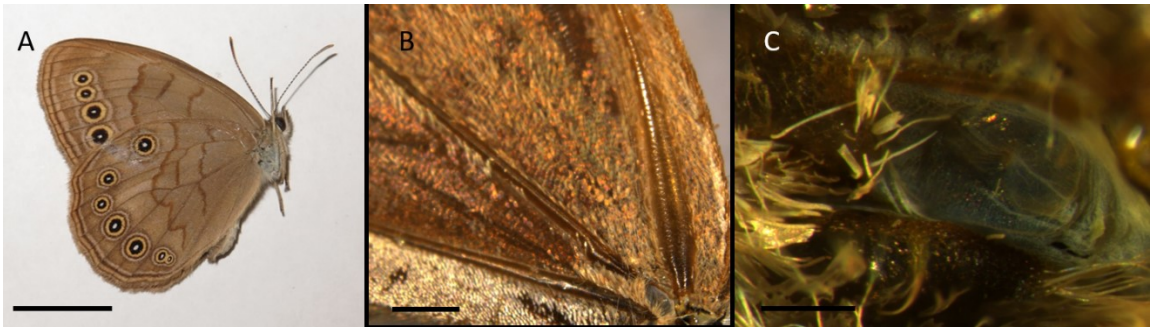


Figure A3.34. *Satyrodes eurydice.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

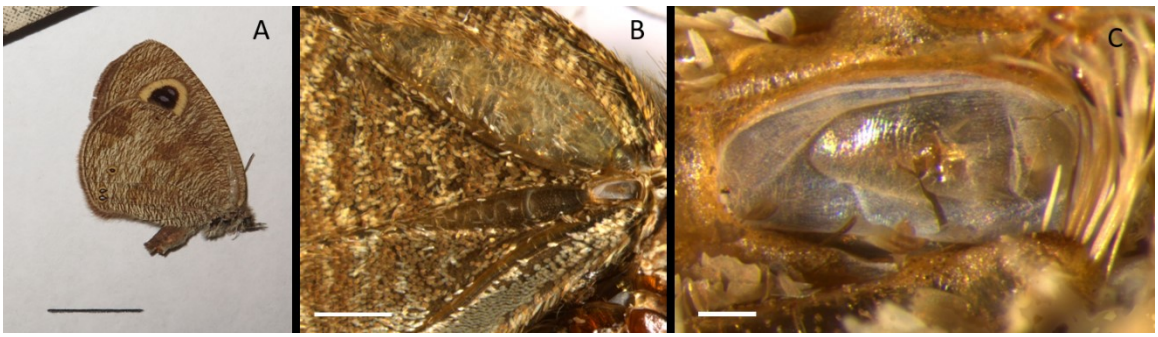


Figure A3.35. *Ypthima albida.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

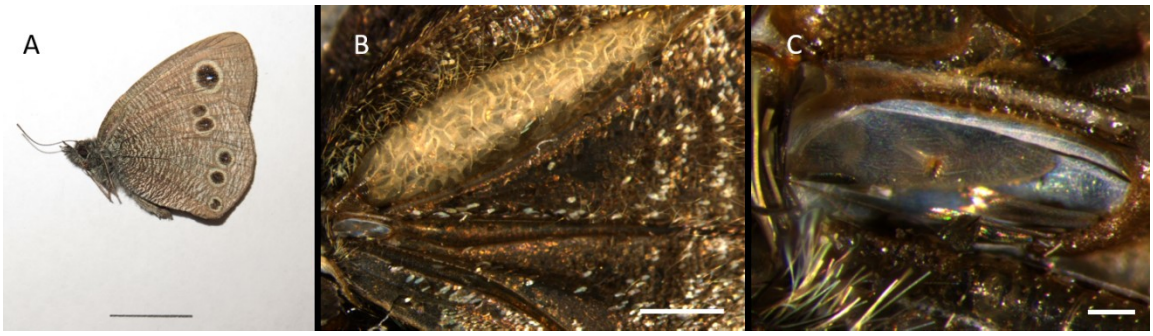


Figure A3.36. *Ypthima baldus.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

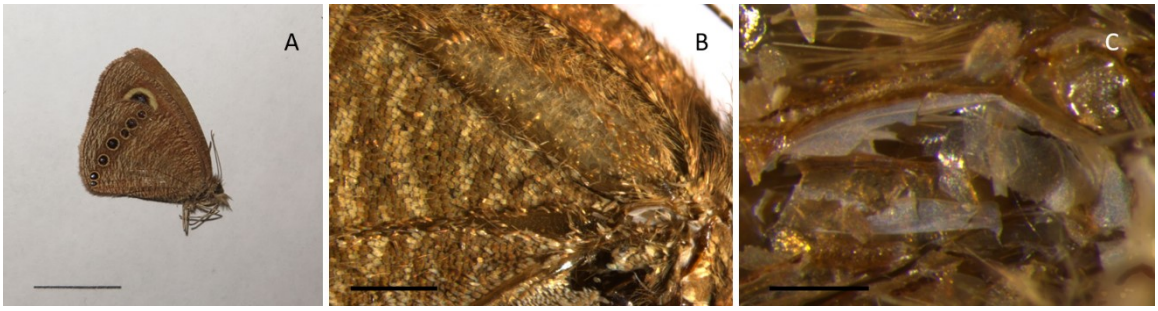


Figure A3.37. *Ypthimomorpha irrorata.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ (damaged from manipulation). Scale bar: 200 µm.

Morphini

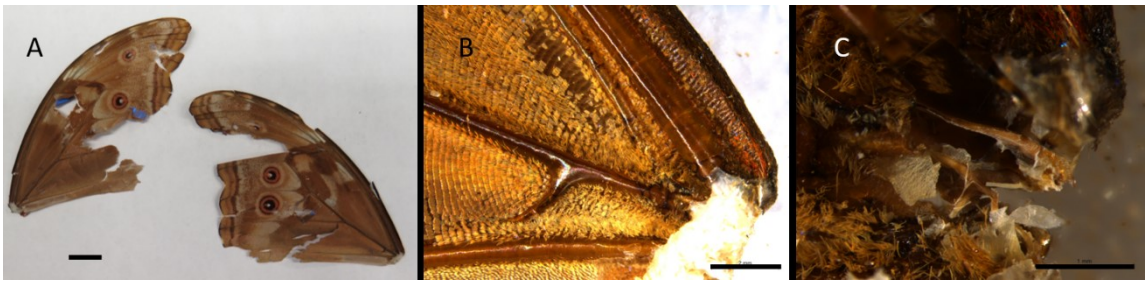


Figure A3.38. *Morpho amathonte.* (A) Forewings of butterfly, ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ (damaged from manipulation). Scale bar: 500 µm.

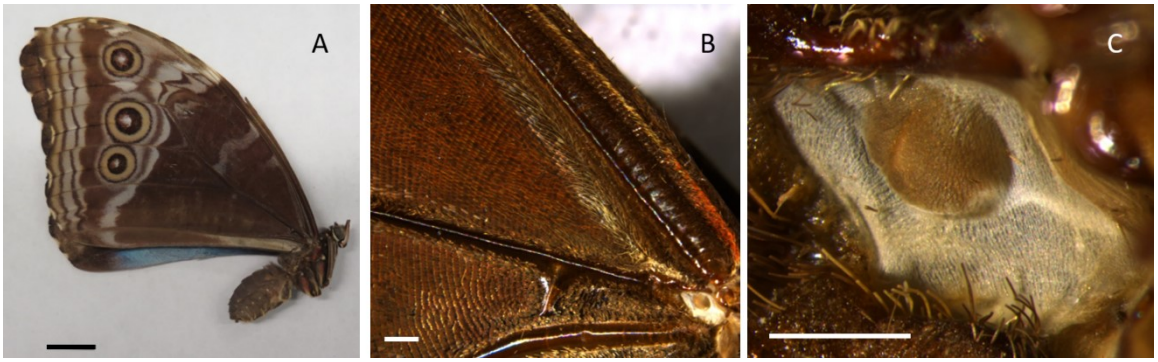


Figure A3.39. *Morpho micropthalmus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

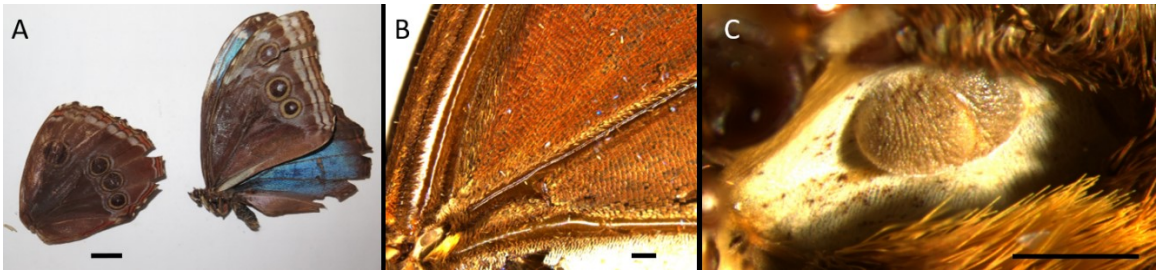


Figure A3.40. *Morpho peleides.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

Brassolini

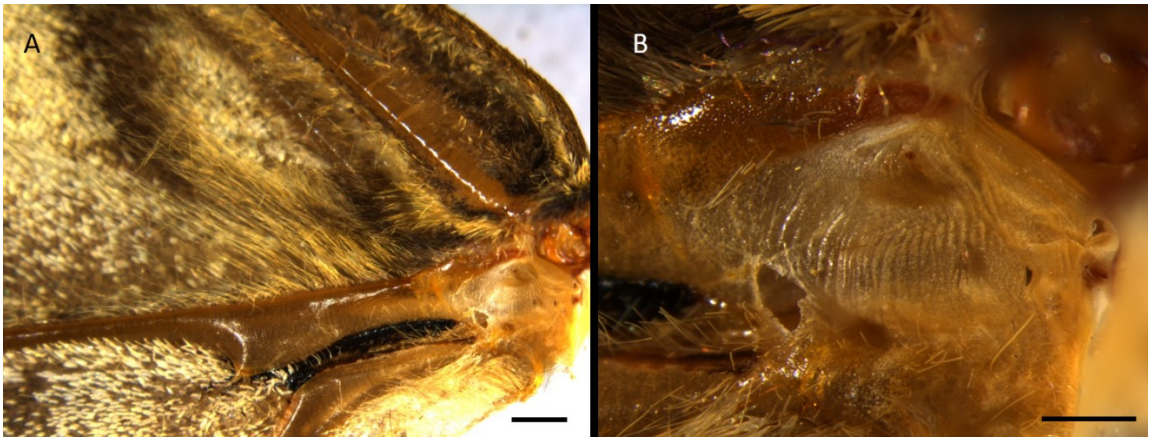


Figure A3.41. *Caligo eurilochus.* (A) Forewing on ventral side, scales removed. Scale bar: 1 mm, (B) Well-developed Vogel's organ. Scale bar: 500 µm.

Figure A3.42. *Caligo ilioneus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

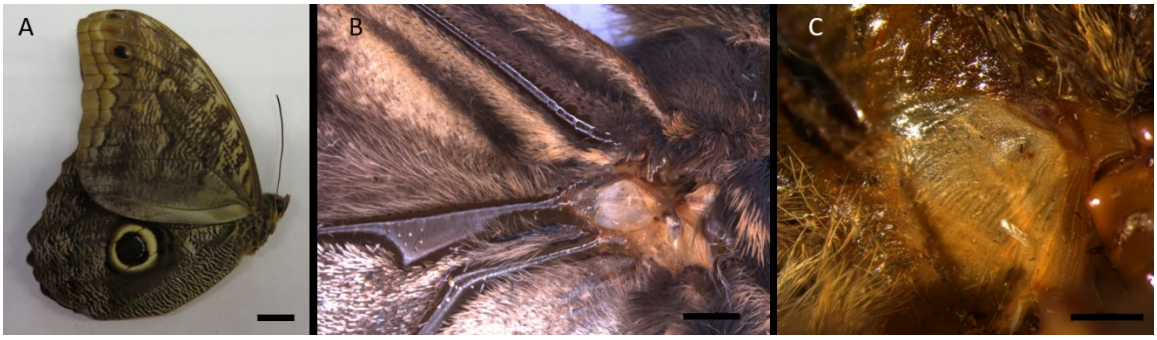


Figure A3.43. *Caligo memnon.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

Elymiini

Figure A3.44. *Elymniosis bammakoo.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

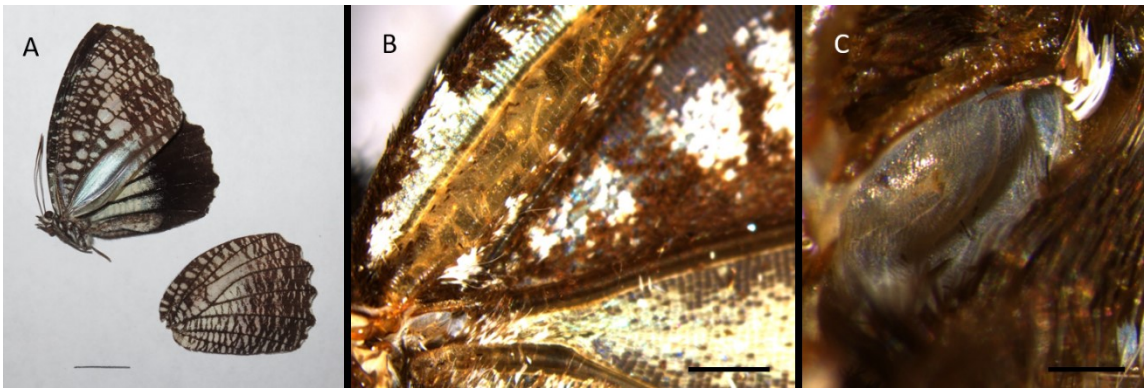


Figure A3.45. *Elymnias kanekei.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

Haeterini

Figure A3.46. *Pierella astyoche.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

Melanitini

Figure A3.47. *Gnophodes betsima.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 100 µm.

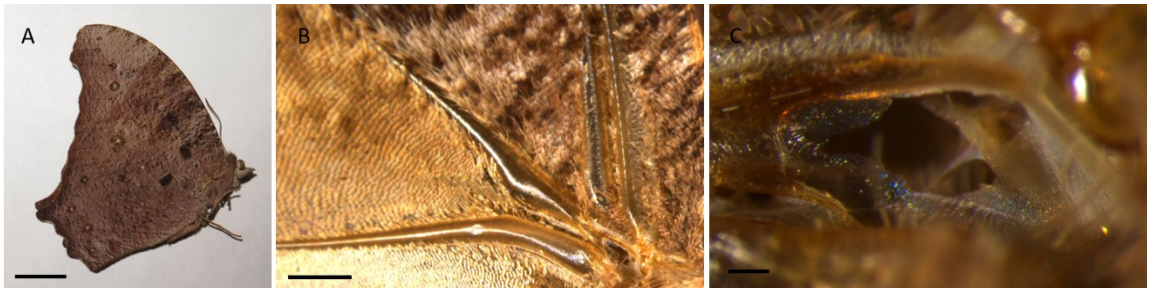


Figure A3.48. *Melanitis leda.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ (damaged by manipulation). Scale bar: 100 µm.

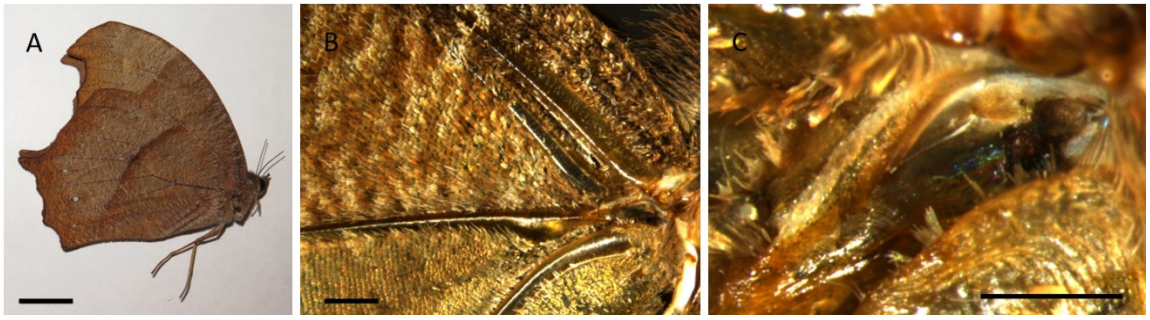


Figure A3.49. *Melanitis leda.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ (damaged by manipulation). Scale bar: 100 µm.

Dirini

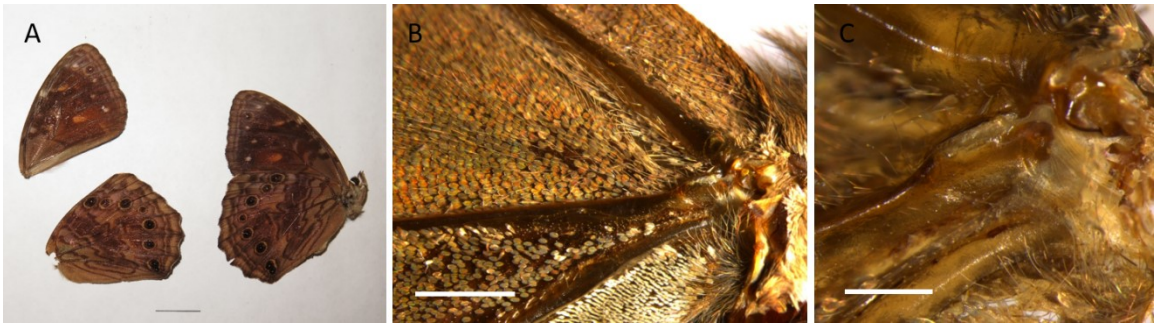


Figure A3.50. *Paralethe dendrophilus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Absent Vogel's organ. Scale bar: 500 µm.

Amathusiini

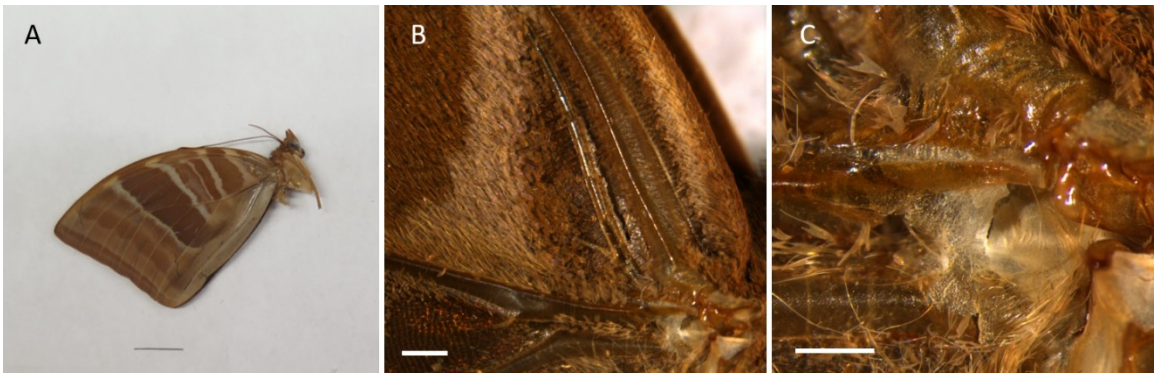


Figure A3.51. *Amathusia phidippus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Uncertain Vogel's organ. Scale bar: 500 µm.

Zetherini

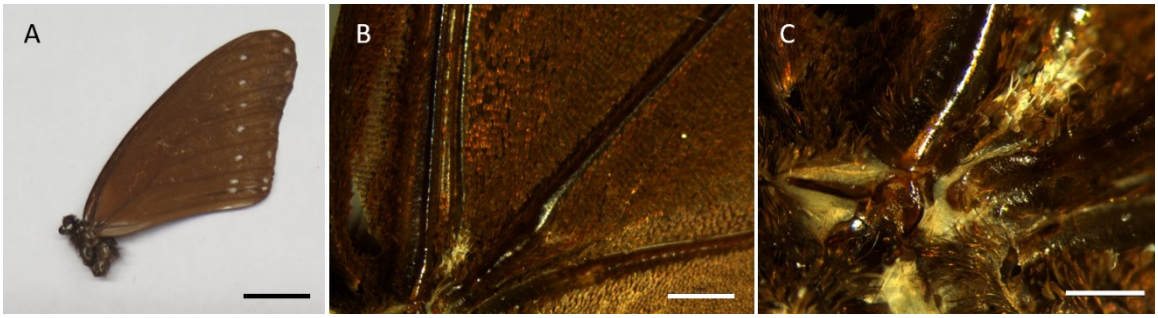


Figure A3.52. *Zethera musa.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Absent Vogel's organ. Scale bar: 500 µm.

A3.2. Charaxinae

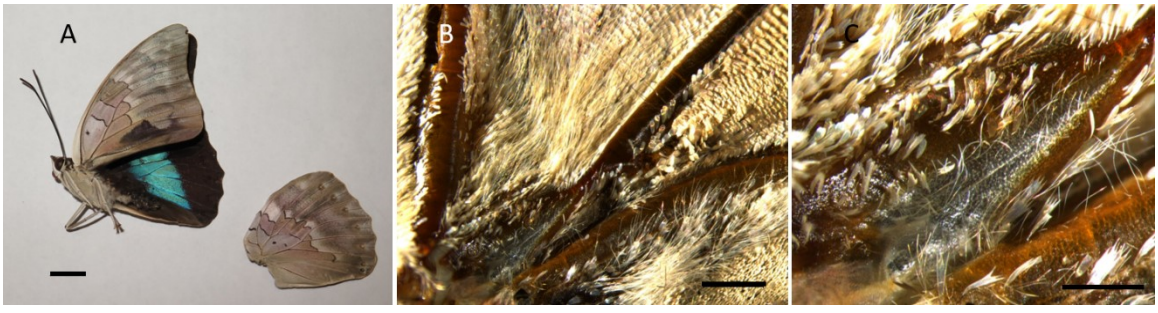


Figure A3.53. *Archaeprepon demophon.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Uncertain Vogel's organ. Scale bar: 500 µm.

Figure A3.54. *Charaxes howarthi.* (A) Whole butterfly on dorsal side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

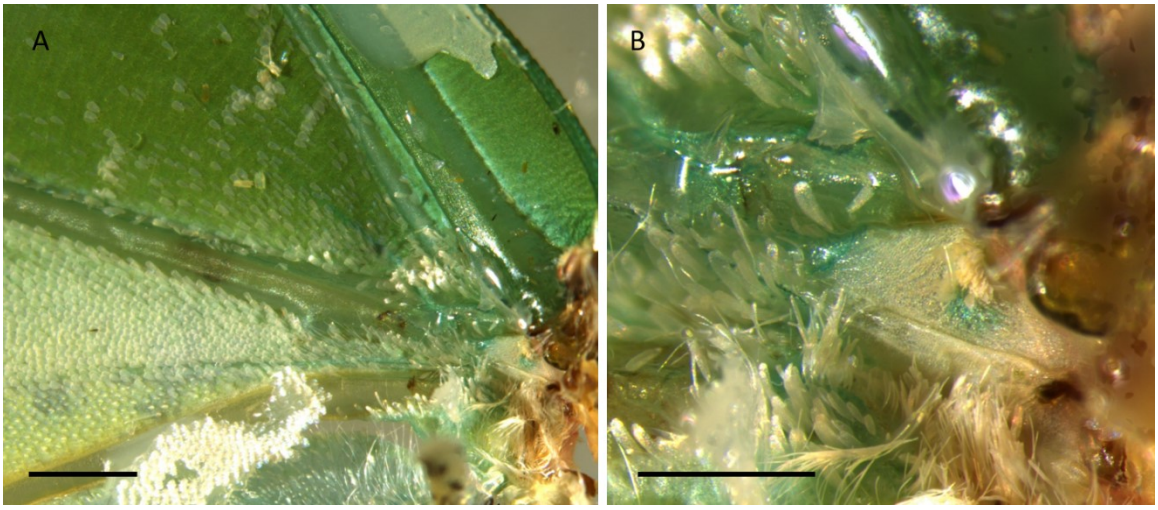


Figure A3.55. *Charaxes subornatus.* (A) Forewing on ventral side, scales removed. Scale bar: 1 mm, (B) Uncertain Vogel's organ. Scale bar: 500 µm.

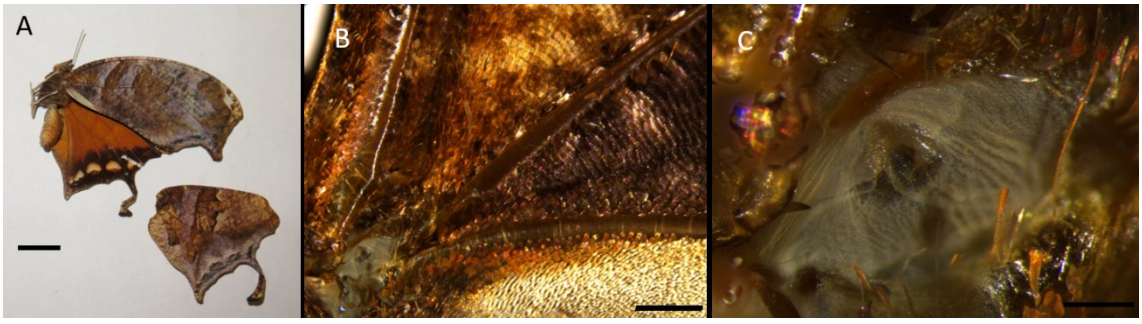


Figure A3.56. *Consul fabius.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 200 µm.

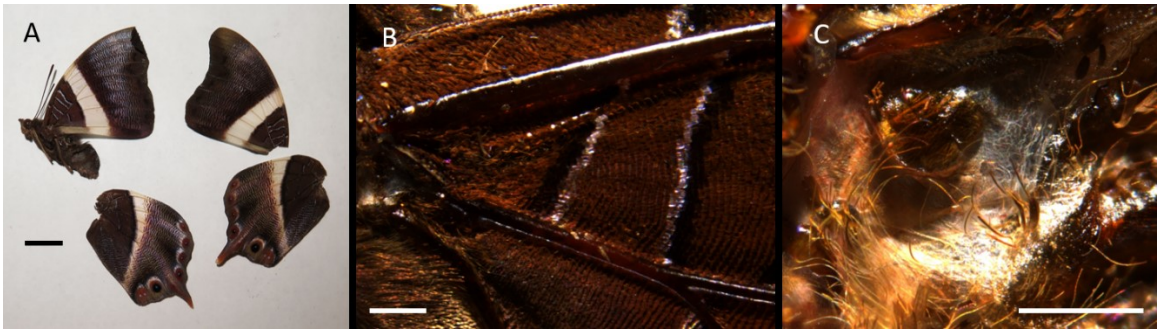


Figure A3.57. *Palla decius.* (A) Whole butterfly on ventral side, hindwings and other forewing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Uncertain Vogel's organ. Scale bar: 500 µm.

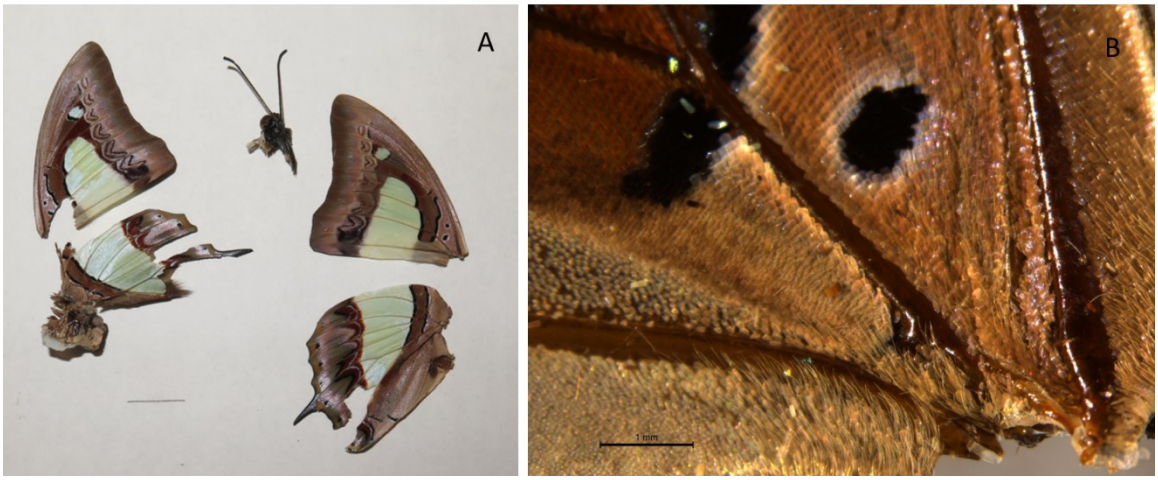


Figure A3.58. *Polyura athamas.* (A) Whole butterfly on dorsal side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Classified in this thesis as well-developed Vogel's organ, but was removed - may need re-evaluation).

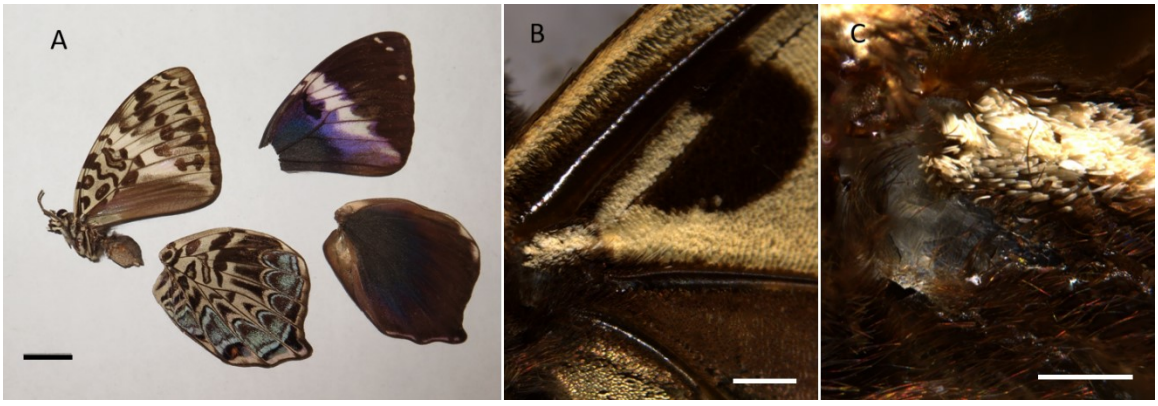


Figure A3.59. *Prothoe franck.* (A) Whole butterfly on ventral side, hindwings and other forewing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Uncertain Vogel's organ. Scale bar: 500 µm.

A3.3. Heliconiinae

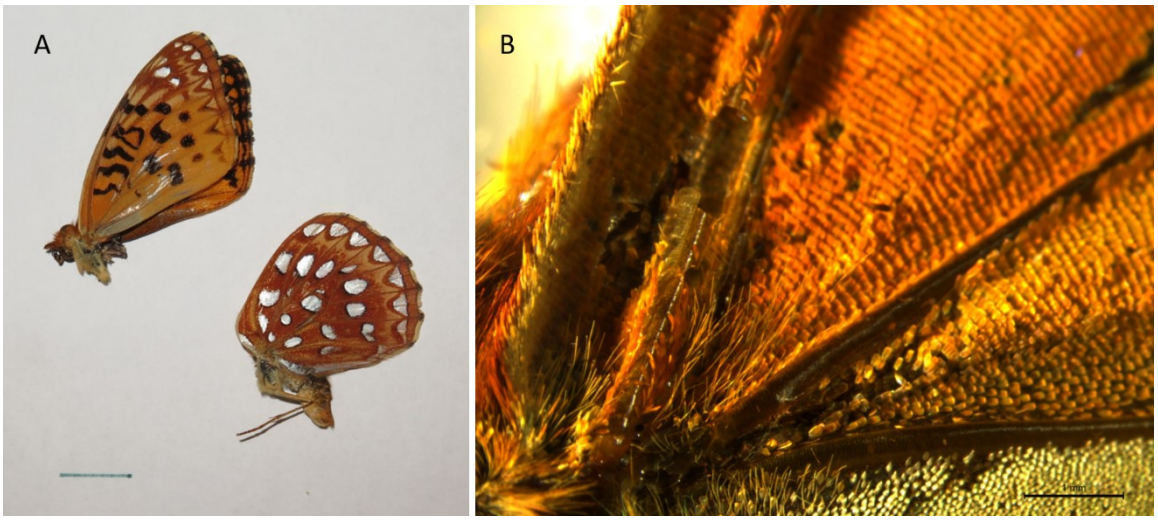


Figure A3.60. *Argynnis aphrodite.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

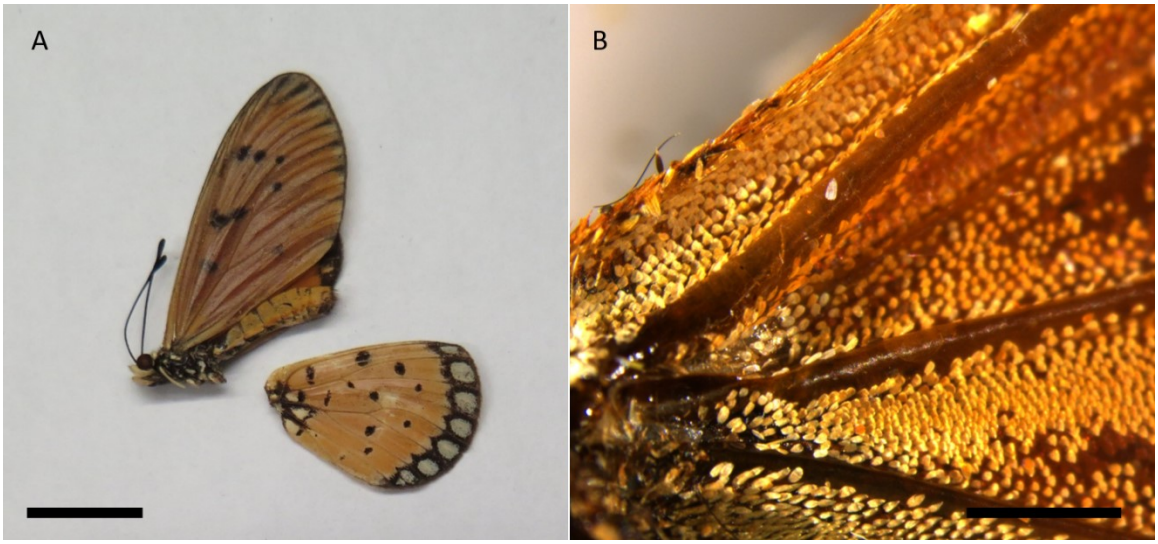


Figure A3.61. *Acraea viola.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

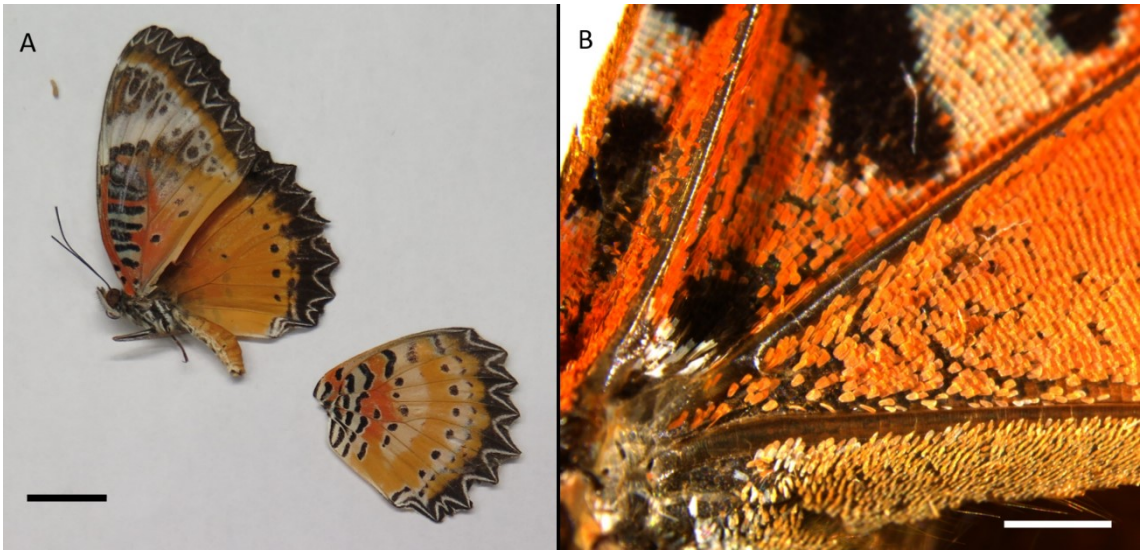


Figure A3.62. *Cethosia cyane.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

Figure A3.63. *Cirrochroa regina.* (A) Forewings on ventral side, only parts available. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

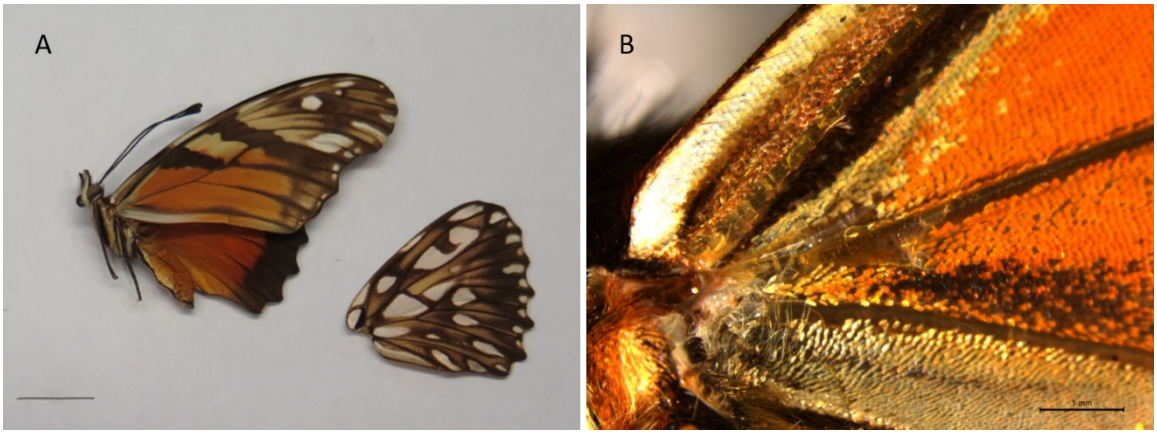


Figure A3.64. *Dione juno.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

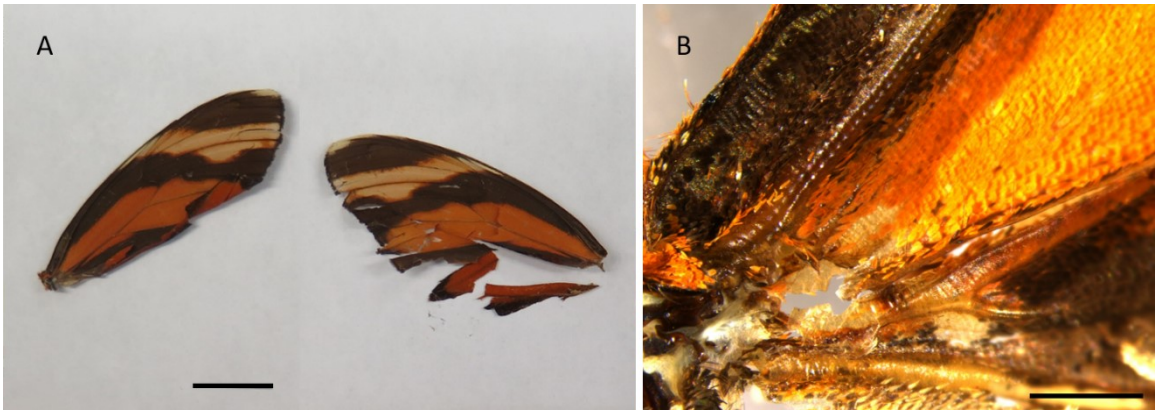


Figure A3.65. *Dryadula phaetusa.* (A) Forewings on ventral side, only parts available. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

Figure A3.66. *Dryas iullia.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

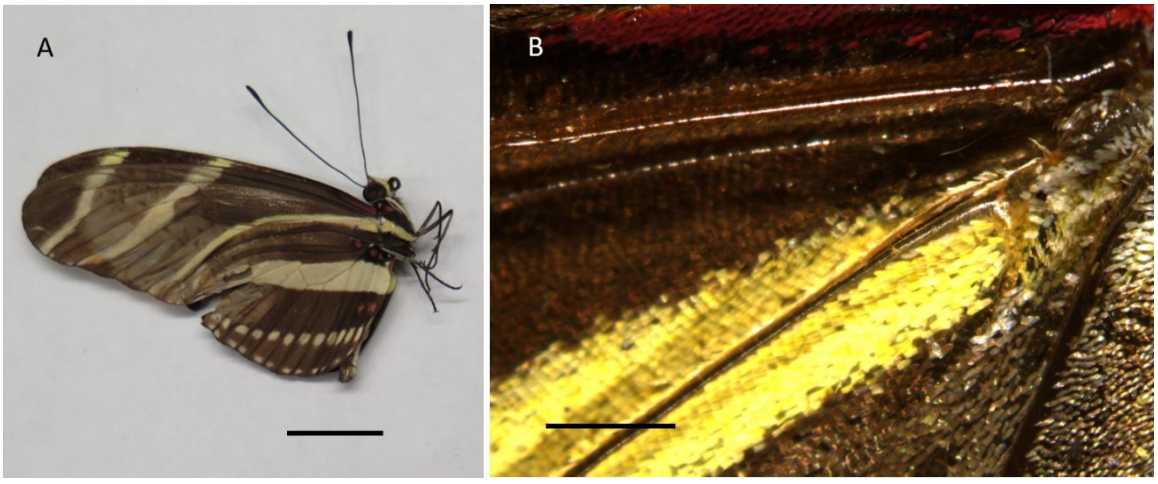


Figure A3.67. *Heliconius charitonia.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

Figure A3.68. *Heliconius erato.* (A) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

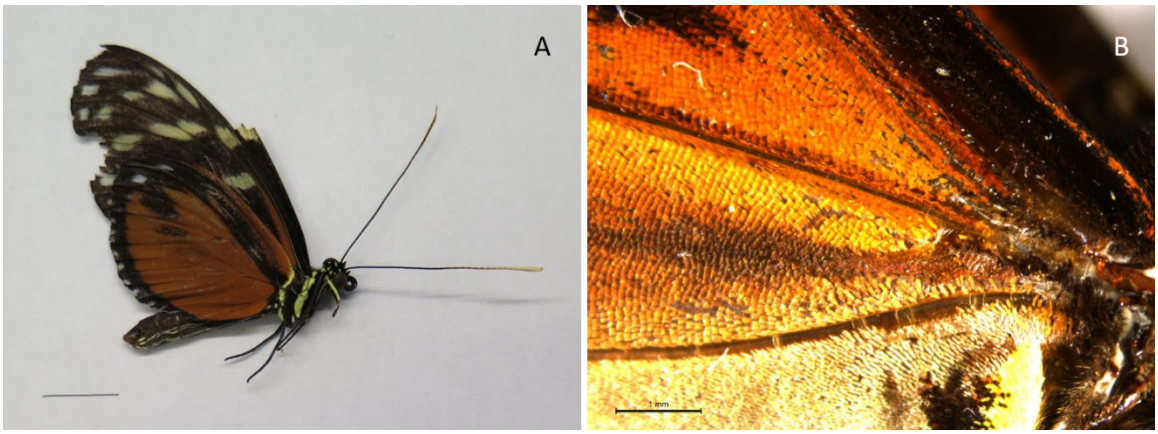


Figure A3.69. *Heliconius hecale.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

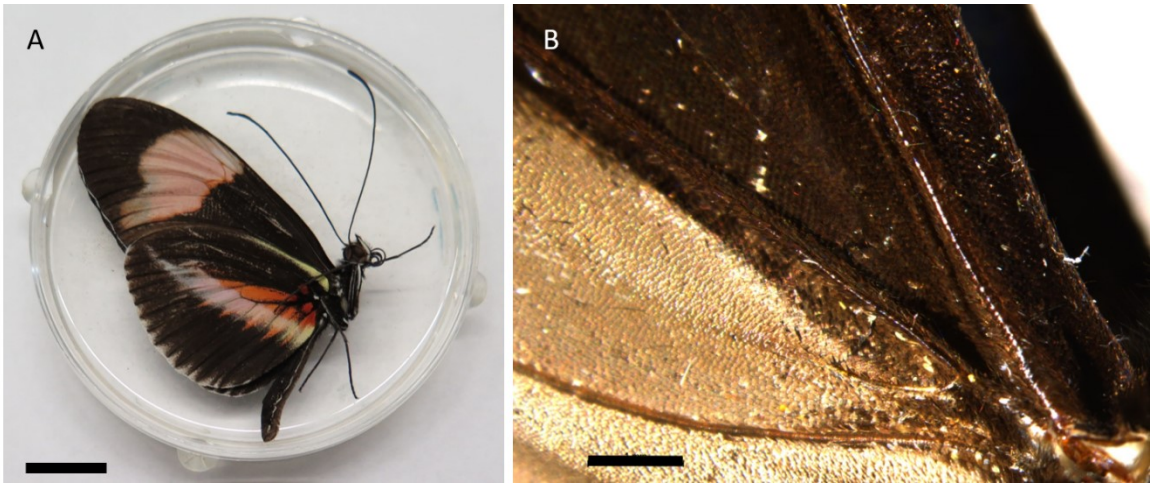


Figure A3.70. *Heliconius melpomene.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

A3.4. Danaiinae

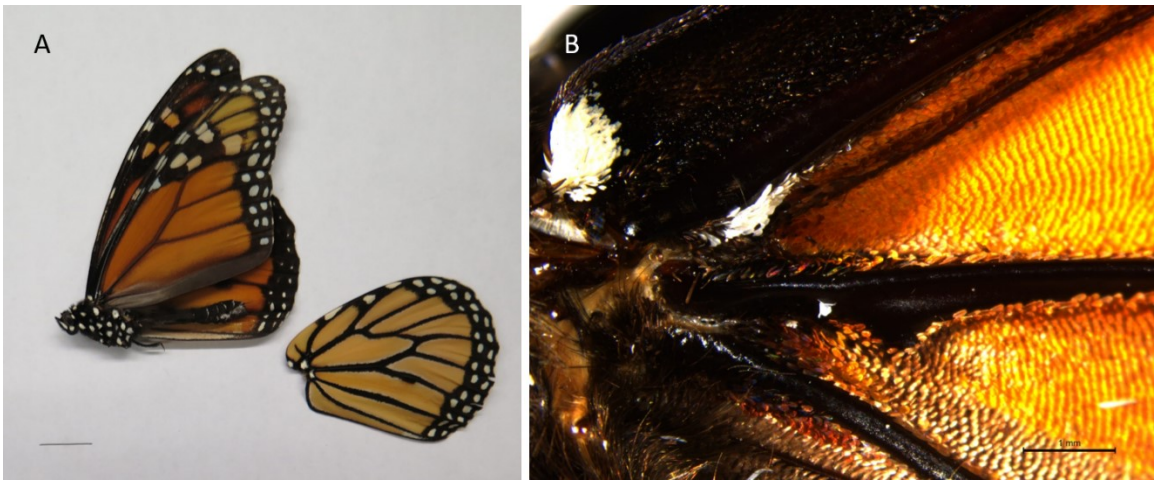


Figure A3.71. *Danaus cleophile.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

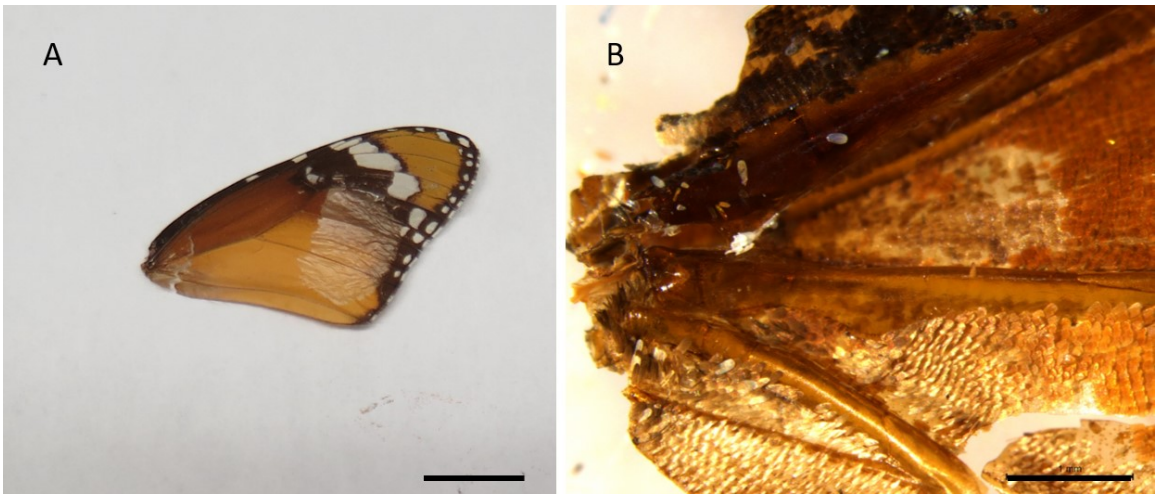


Figure A3.72. *Danaus chrysippus.* (A) Forewing on ventral side, only body part available. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

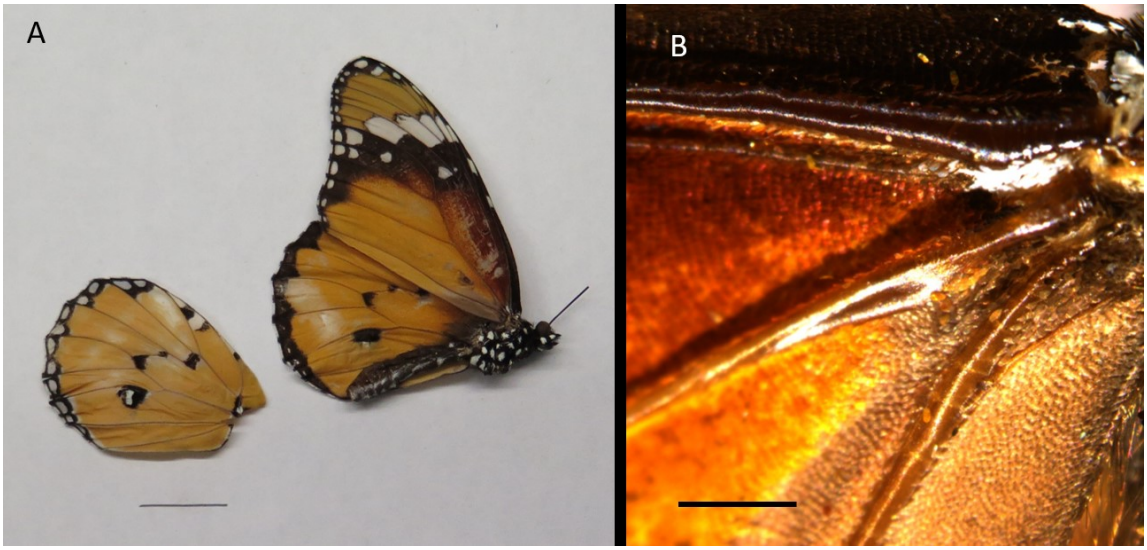


Figure A3.73. *Danaus petilia.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

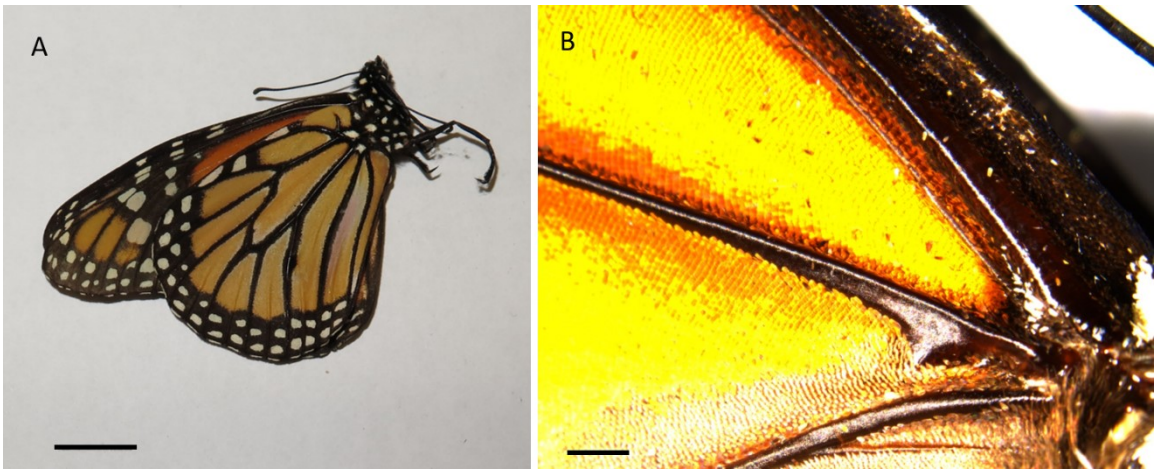


Figure A3.74. *Danaus plexippus.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

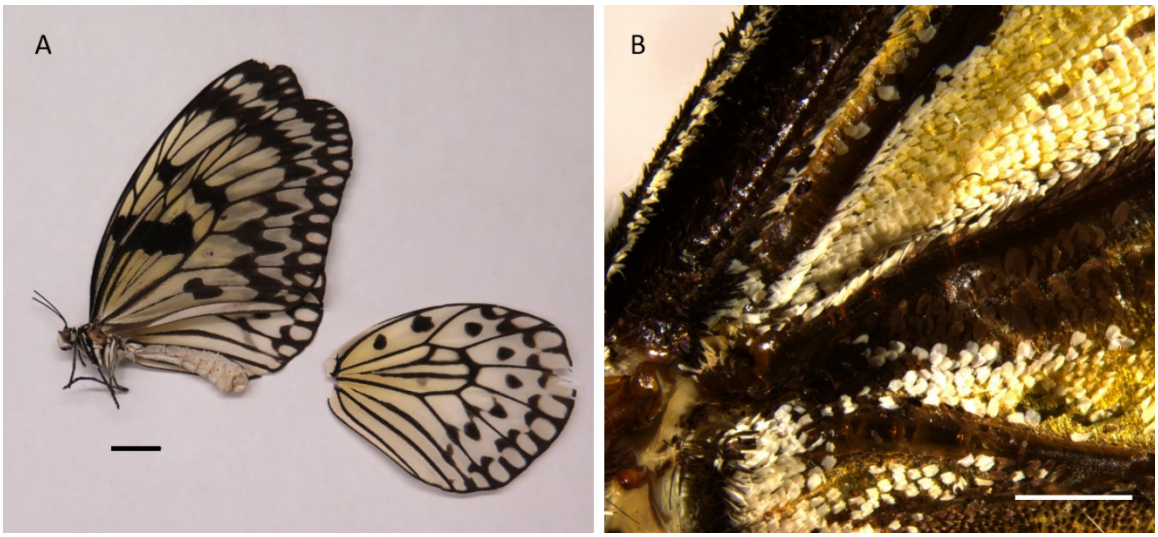


Figure A3.75. *Idea leuconoe.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

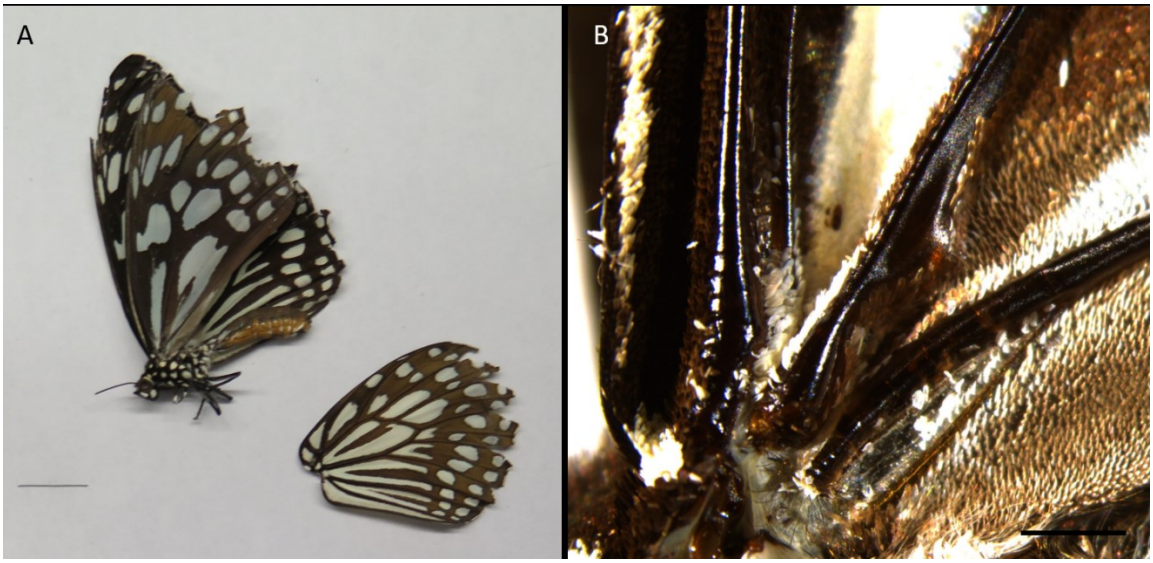


Figure A3.76. *Tirumala limniace.* (A) Whole butterfly on ventral side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm. (Absent Vogel's organ).

A3.5. Nymphalinae

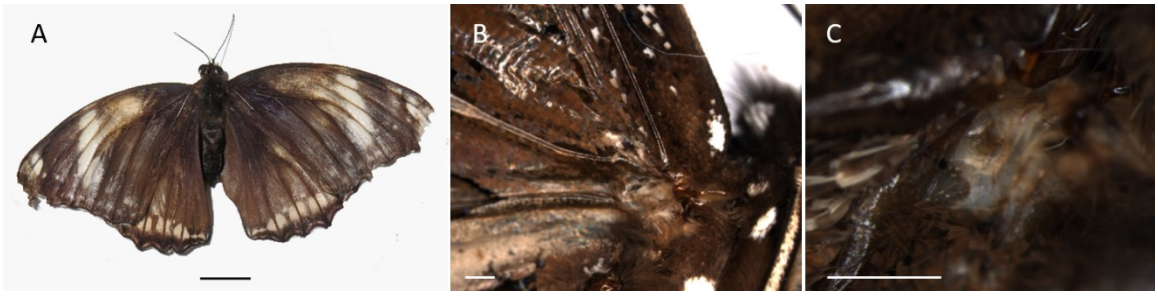


Figure A3.77. *Hypolimnas bolima.* (A) Whole butterfly on dorsal side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Uncertain Vogel's organ. Scale bar: 500 µm.

A3.6. Biblidinae

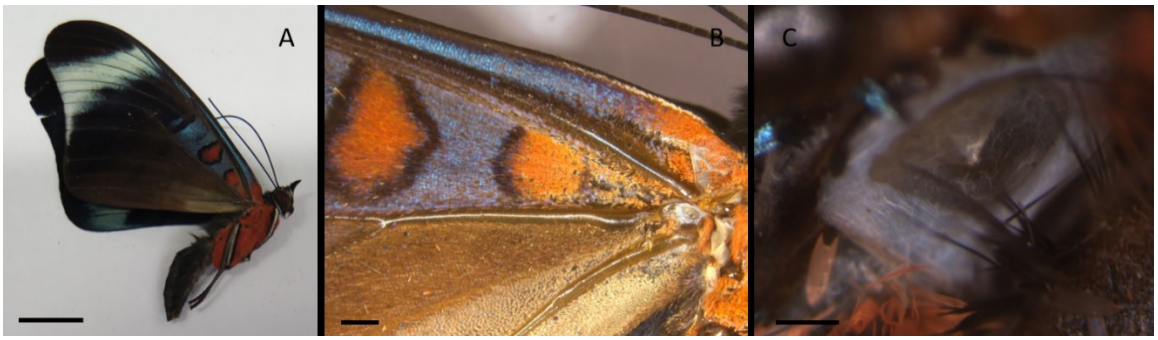


Figure A3.78. *Panacea prola.* (A) Whole butterfly on dorsal side, hindwing removed. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 1 mm.

A3.7. Apaturinae

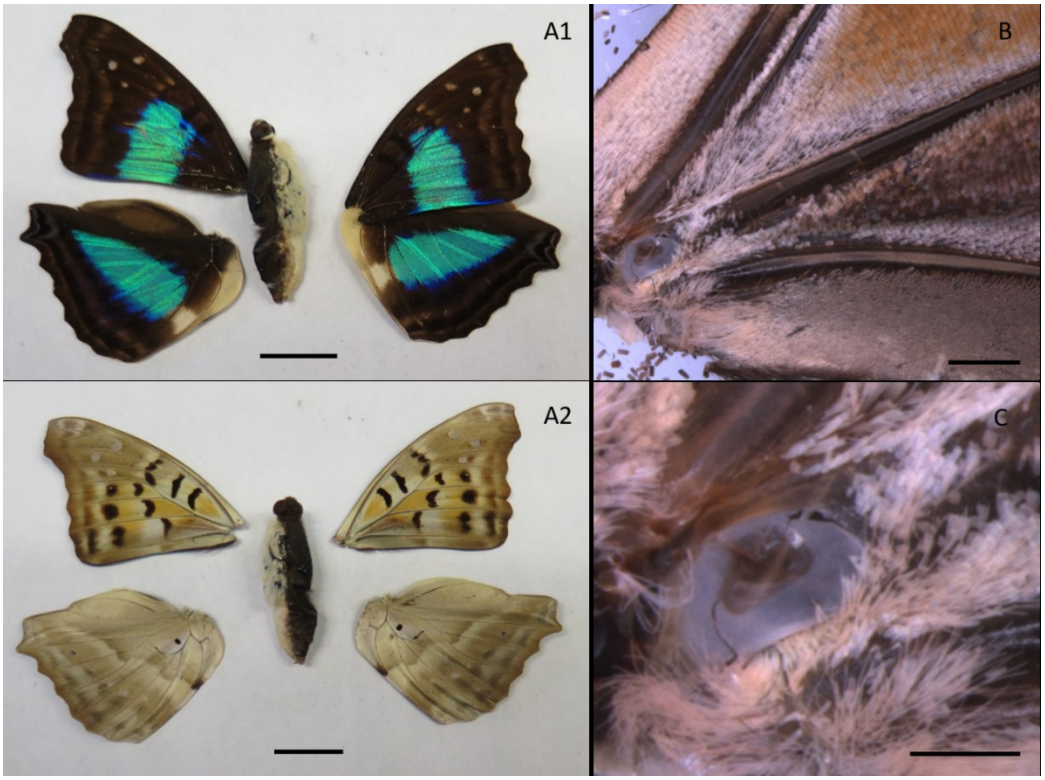


Figure A3.79. *Doxocopa pavon.* (A1) Whole butterfly on dorsal side. Scale bar: 10 mm, (A2) Whole butterfly on ventral side. Scale bar: 10 mm, (B) Forewing on ventral side, scales removed. Scale bar: 1 mm, (C) Well-developed Vogel's organ. Scale bar: 500 µm.

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