Hearing in the Speckled Wood Butterfly, *Pararge aegeria*

(Nymphalidae: Satyrinae)

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Abstract

Research on butterfly communication has focused primarily on vision and chemoreception, while the role of hearing remains largely unexplored. The first objective of this study was to investigate the morphology, physiology, and function of the Vogel's organ in *Pararge aegeria* (Satyrinae). Vogel's organ occurs at the base of the forewing and features the typical morphology of a tympanal hearing organ. Physiological recordings of the tympanal nerve reveal broadband sensitivity in the range of 1 kHz – 18 kHz. A best frequency was found at 6.5 kHz (best threshold at 56 dB SPL and median threshold at 70.4 dB SPL). In conjunction with analysis of bird flight sounds, the results support the hypothesis that this Vogel's organ functions to detect avian predators. Secondly, the morphology and taxonomic distribution of Vogel's organ was explored in Satyrinae. Vogel's organ was found throughout this subfamily and a diversity of morphological forms implies functional diversity.

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used in the comparative study including the Canadian National Collection of Insects & Arthropods, National Museum of Natural History (Smithsonian Institution), and the Florida Museum of Natural History (McGuire Center for Lepidoptera and Biodiversity).

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1. Introduction

Butterflies are found at the forefront of evolutionary, ecological, and behavioural research. One area of interest that has received considerable attention is the sensory systems of butterflies. Learning about the sensory system of an animal has important implications for understanding its behaviour and relationship to the environment. At present, the sensory modalities of butterflies for which significant information exists are vision and chemoreception. The highly developed visual system encompasses a broadband wavelength range including our own spectral range and extends into the ultraviolet (Warrant *et al.*, 2003). As butterflies are predominantly day-active, a sense of vision is arguably their dominant sense, playing an integral role in orientation, foraging, host plant identification (Warrant *et al.*, 2003), and mate recognition (Rutowski & Warrant, 2002). In addition, butterflies have numerous sense organs and glands located all over their body, which facilitate transmission of information regarding taste and smell (chemoreception) (Hallberg & Poppy, 2003). Chemoreception provides cues to ovipositing female butterflies regarding decisions of host plant selection (Ono & Yoshikawa, 2004) and plays a role in the discrimination of individual butterflies (Takanashi *et al.*, 2001). To date, most butterfly research has focused on vision and chemoreception. A sense of hearing, which is well developed in many insects (Yager, 1999; Yack, 2004), has yet to be thoroughly explored in butterflies.

Butterflies comprise 3 of the 46 superfamilies of Lepidoptera (Fig.1). There are two primarily diurnal superfamilies, the Hesperoidea and the Papilionoidea, and a nocturnal superfamily, the Hedyloidea, found only in the Neotropics. The remaining Lepidopteran superfamilies are called moths (Kristensen & Skalski, 1998). In contrast to

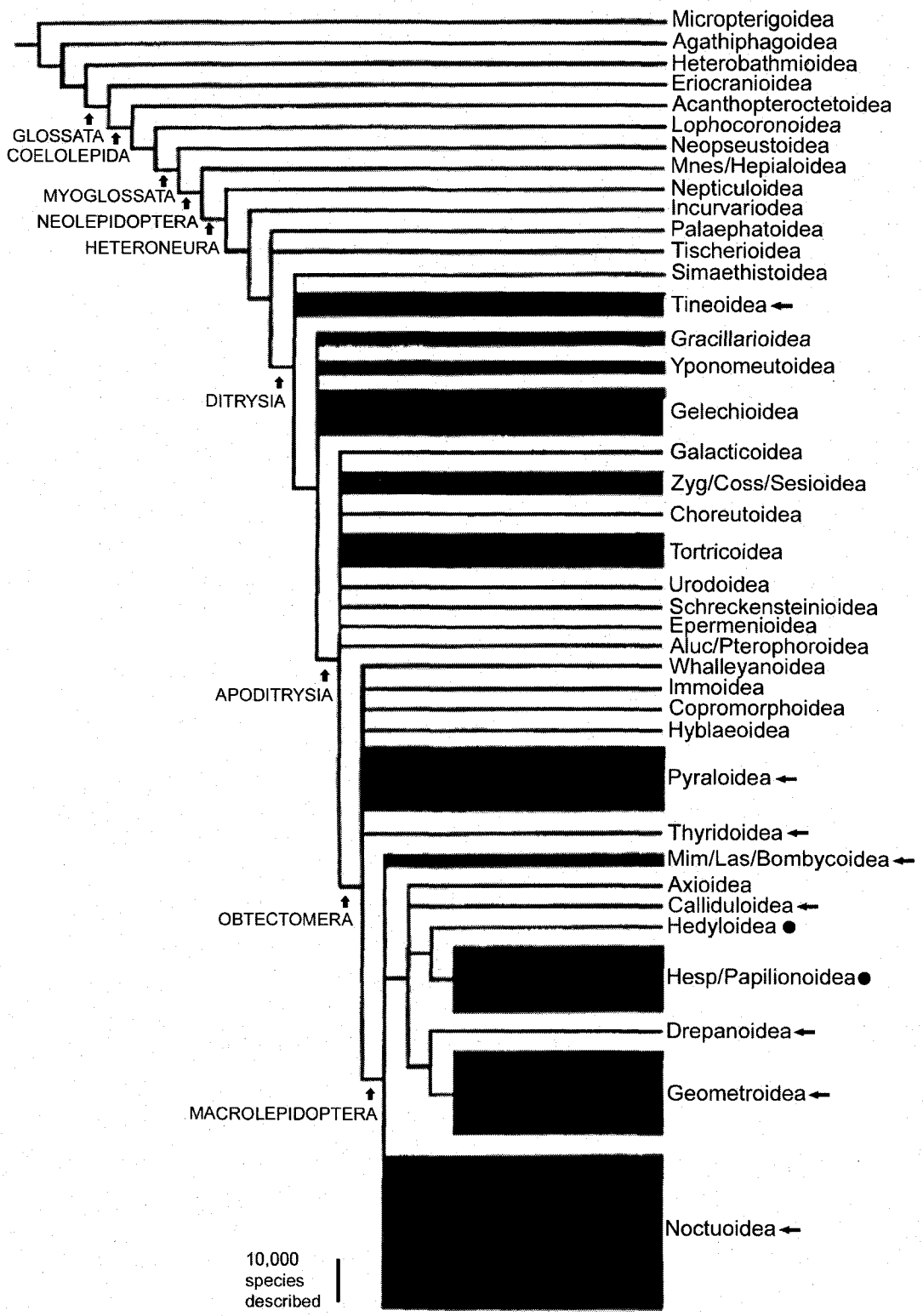

Fig. 1. Phylogenetic hypothesis for Lepidopteran superfamilies. Butterfly superfamilies are indicated with filled circles. Moth superfamilies with a sense of hearing are indicated with arrows. Reproduced from Kristensen & Skalski (1998).

butterflies, the majority of moths are nocturnal, exposing them to predation by insectivorous bats. At least 8 moth superfamilies are equipped with the ability to hear, with tympanal ears residing on the thorax, abdomen, mouthparts, and wings. Primarily, moths use this acoustic sense to detect the ultrasonic cries of the bats. In addition, some moth groups have secondarily evolved the ability to produce sound and in these cases, hearing also functions for sexual communication (Conner, 1999; Minet & Surlykke, 2003).

Until recently butterflies were not considered to have a sense of hearing, but recent evidence has caused us to reconsider. For example, in the Hedyloidea, hearing is now thought to occur throughout the group. Hedylid butterflies have ultrasound sensitive ears on their wings believed to function as bat detectors (Fig. 2A,B) (Yack & Fullard, 2000; Yack *et al.*, submitted). These uniquely designed tympanal ears are located at the base of the forewing (Fig. 2A), where a thin tympanal membrane sits within a tympanic cavity formed by swelling of the subcostal vein (Fig. 2B). Stimulation with high-intensity ultrasound elicits evasive flight maneuvers, in a manner similar to moths that detect and evade bats. The revelation of hearing in this intriguing group has important implications for the evolution of hearing in butterflies, since hedylids are suggested to represent the "living ancestors" of the Hesperoidea and Papilionoidea butterflies (Scoble, 1995; Yack & Fullard, 2000; Minet & Surylkke, 2003).

There is also increasing evidence that hearing exists in a large family of the Papilionoidea, the Nymphalidae (Fig. 3). Anatomical evidence stems from reports of Vogel's organ, first reported by Richard Vogel in the early 1900's. Vogel's organ is an ovoid membrane located at the base of the forewing in some nymphalid butterflies

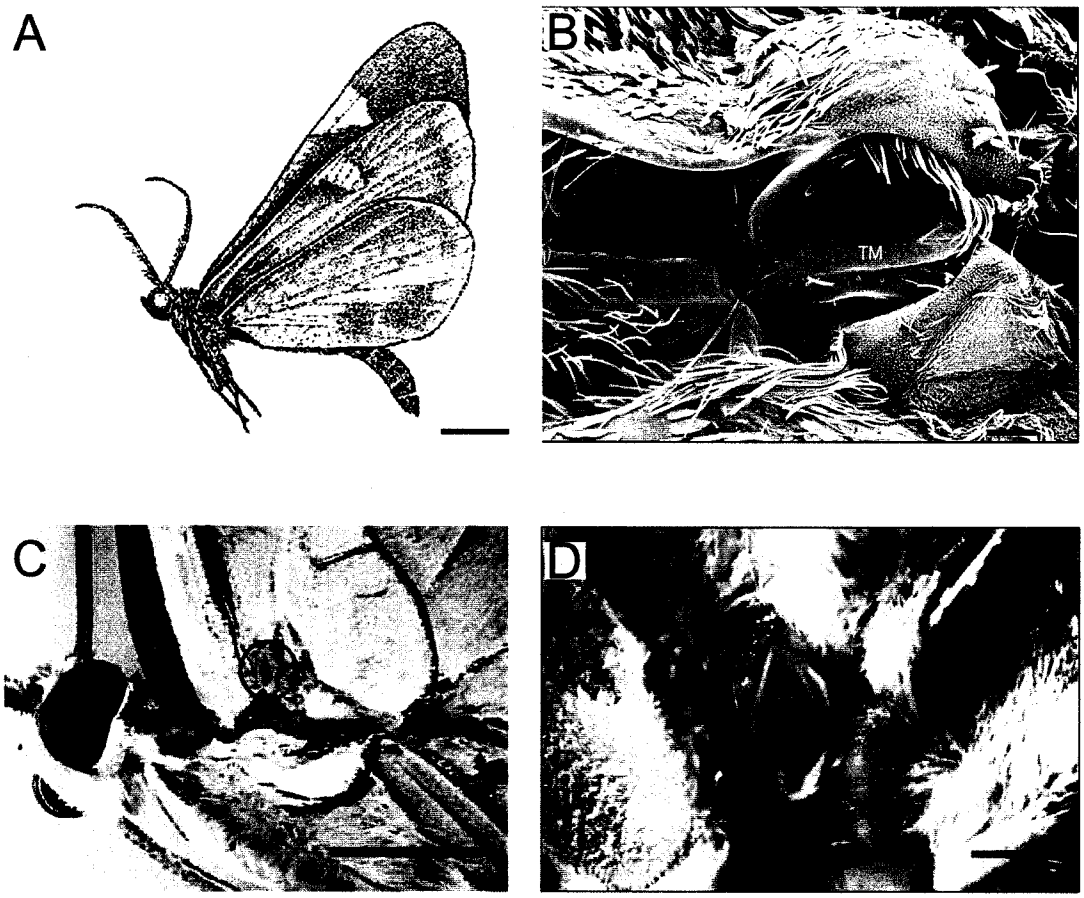

Fig. 2. Two experimentally confirmed cases of hearing in butterflies. (A) Lateral view of a hedylid butterfly, *Macrosoma heliconaria,* with an arrow indicating the position of the ear on the forewing. Scale bar, ~3mm. (B) SEM of the tympanal ear of *M. heliconaria*. TM, tympanal membrane. Scale bar, 120 µm. (C) View of the ventral surface of the left forewing of *Hamadryas feronia* with the Vogel's organ circled. Scale bar, 3mm. (D) Close-up of the Vogel's organ in *H. feronia*. Scale bar, 200µm. A&B reproduced from Yack & Fullard (2000). C&D reproduced from Yack *et al.* (2000).

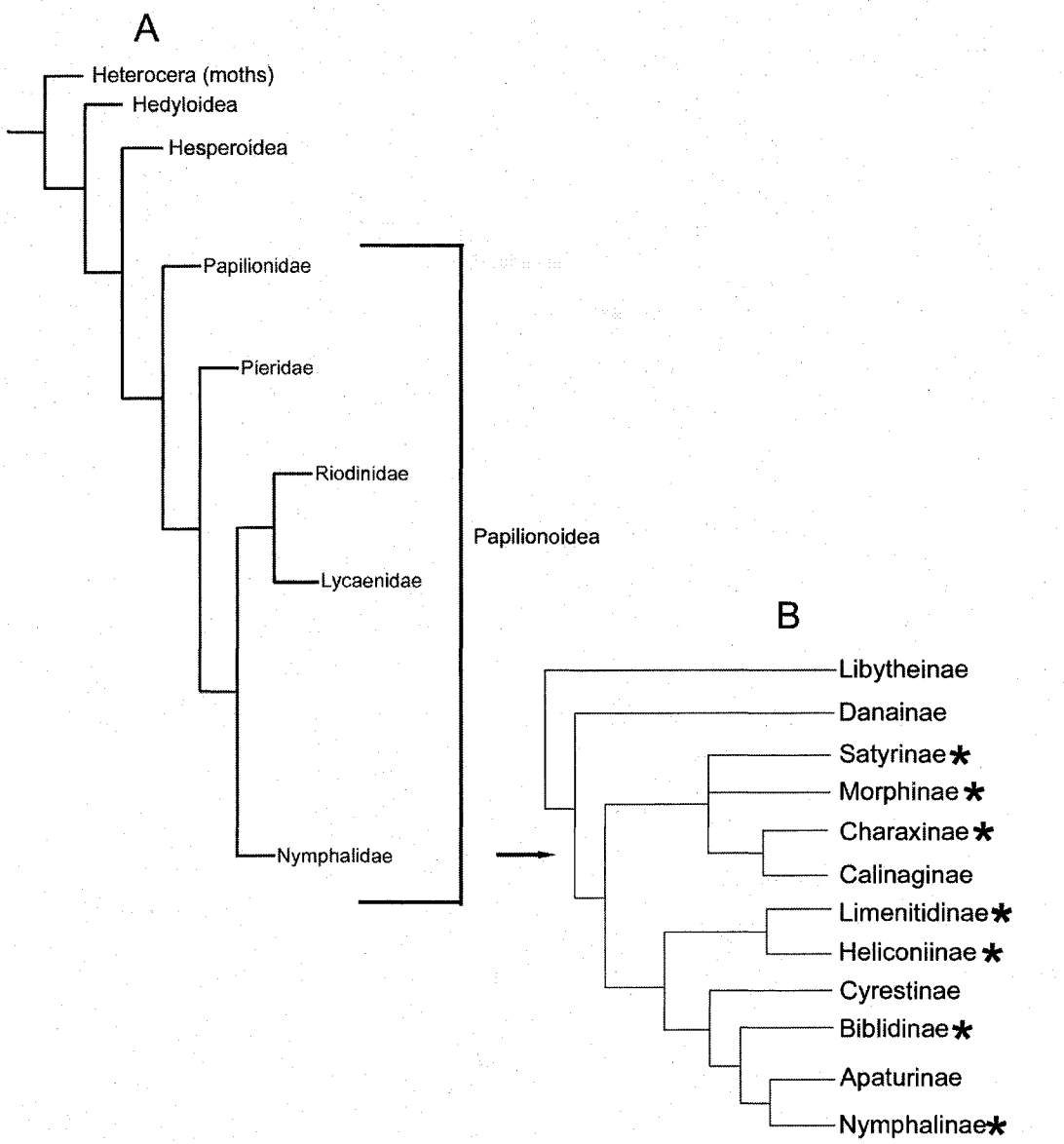

Fig. 3. Proposed phylogenetic relationships of the butterflies (Rhopalocera). (A) Phylogeny of the butterfly families based on combined morphological and molecular data sets. Adapted from Wahlberg *et al.* (2005). (B) Phylogeny of the family Nymphalidae based on molecular data. Adapted from Wahlberg *et al.* (2003). Subfamilies where sound production and/or hearing has been suggested are denoted with an asterisk (*).

(Vogel, 1912; LeCerf, 1926; Bourgogne, 1951). Vogel suggested the organ may have an auditive function based on its anatomical resemblance to tympanal ears in Orthoptera. Otero (1990a) expanded on early anatomical work to include several nymphalid species, proposing that this character is widespread throughout Nymphalidae and variable in form – from absent to well-developed. Most reports of Vogel's organ have focused on external anatomy. However, direct neuromorphological, physiological, or behavioural evidence for its role in hearing is generally lacking.

There have only been two published studies testing the function of hearing in butterflies using physiological methods. In the most comprehensive study of butterfly hearing to date, *Hamadryas feronia* (Biblidinae) was found to possess a well-developed Vogel's organ (Fig. 2C,D) and produce audible clicking sounds. Yack *et al.* (2000) demonstrated that *H. feronia* is sensitive to low-frequency sounds with a best frequency at 1.75 kHz (median threshold at 68 dB SPL). Hearing was found to be facilitated by the Vogel's organ, rather than the membranous ampulla as once advocated by Swihart (1967). The sounds produced by *H. feronia* have a peak energy around 13-15 kHz (80-100 dB SPL at 10 cm), and they present sufficient energy to be detected by *H. feronia*. A sense of hearing in this species is suggested to function in conspecific communication.

Swihart (1967) was one of the first to investigate a physiological aspect of butterfly hearing. Using *Heliconius erato* (Heliconiinae), whole-nerve recordings from the IIIN1c hindwing nerve demonstrated sensitivity to low-frequency sound stimuli at a best frequency of 1.2 kHz at 61 dB SPL. *Heliconius* does not possess a Vogel's organ, however there is recent evidence that it produces sound (Hay-Roe, 2004). Swihart (1967)

also recorded from the IIN1c forewing nerve of *Hamadryas* sp. and similar sensitivity was demonstrated. However, a hearing organ was not confirmed at that time.

Behaviour is another important line of evidence for establishing a sense of hearing in butterflies. Ribaric & Gogala (1996) reported what they believed to be an acoustic startle response to low-frequency sounds (125 Hz – 16 kHz) in *Erebia manto* and *E. euryale* (Satyrinae). Behaviourally, *Erebia* responded best to 1 kHz sounds with a lowest threshold of 49 dB SPL. In the field, congregations of these butterflies were found to demonstrate responses to low-frequency sounds in the form of wing movements and escape flight. Hearing in *Erebia* was suggested to function in the detection of bird vocalizations, but this hypothesis was not tested. Behavioural responses have also been shown in the crepuscular *Manataria maculata* (Satyrinae). At dusk and dawn, ultrasonic stimulation (26 kHz, 110 dB SPL) elicits evasive flight maneuvers suggesting an adaptation for bat detection. *M. maculata* do possess a Vogel's organ but its role in hearing was not investigated (Rydell *et al.*, 2003).

In addition, there are several preliminary reports based on either external anatomical or behavioural observations supporting the notion that hearing and sound production is widespread in nymphalid butterflies (Table 1). Despite an abundance of indirect evidence, much work remains with respect to the study of hearing in Nymphalidae. Given the purported morphological variation of Vogel's organ, it is likely that Vogel's organ supports a diversity of functions, or in some cases, no function at all. Conclusive studies including anatomical, physiological, and behavioural evidence are required in order to confirm the role of hearing in different species.

Table1. Suggested and/or confirmed evidence of hearing and sound production in butterflies, not including results of this study.

	Evidence for Sound Production		Evidence for Hearing		
	Description	Reference	Anatomical[1]	Response to Sound (B = Behavioural) (P = Physiological)	Reference
Papilionoidea					
Nymphalidae					
Biblidinae					
Hamadryas (=Ageronia) sp.	Clicking	4,12,47,56,79,105	VO	Yes (B)	12,45,47
Hamadryas feronia	Clicking,Crackling	16,20,31,34,45,46,47,56,77,99	VO	Yes (P)	47,55,77,99
Hamadryas februa	Clicking,Crackling	34,50,104	VO	----	47
Hamadryas fornax	Clicking,Crackling	7,31,34	----	----	
Hamadryas amphinome	Clicking,Crackling,Grating	20,31,34,46,47,52,77	VO	----	47,77
Hamadryas arethusa	Clicking	31	----	----	
Hamadryas ferentia	Clicking	7,20	----	----	
Hamadryas belladonna	Crackling	34	----	----	
Hamadryas epinome	Crackling	34	----	----	
Hamadryas guatemalena	Clicking,Crackling	34,46,51	VO	----	47
Hamadryas amphichloe	Clicking	34	----	----	
Hamadryas laodamia	Yes[2]	34	----	----	
Hamadryas glauconome	Yes[2]	46	VO	----	47
Limenitidinae					
Limentis (=*Basilarchia*) sp.	Yes[2]	14	----	----	
Nymphalinae					
Vanessa sp.	Hissing	31,88	----	----	
Vanessa urticae	Buzzing,Grating,Rasping,Hissing	19,44,74,78	----	----	
Vanessa polycholoros	Buzzing,Grating,Rasping,Hissing	19	----	----	
Vanessa antiopa	Buzzing,Grating,Rasping,Hissing	19,20,36,74,80	----	----	
Vanessa (=*Neptis*) *io*	Hissing,Clicking,Squeaking	3,20,28,44,67,74,78,89	----	----	
Neptis hyla	Clicking	70	----	----	
Eunica margarita	Clicking	31	----	----	
Colobura (=*Dirce*) *dirce*	Clicking	38	----	----	
Satyrinae					
Pharneuptychia pharnaba	Clicking	37	----	----	
Erebia euryale	----	----	VO	Yes (B)	60
Erebia manto	----	----	VO	Yes (B)	60
Manataria maculata	----	----	----	Yes (B)	65
Epinephele jurtina	----	----	VO	----	4,83
Coenonympha pamphilus	----	----	VO	----	4,83

Cercyonis pegala	----		VO	Yes (B)	25
Cercyonis alope	----		VO	Yes (B)	25
Euptychia terrestris	----		VO	----	41,55
Melanargia sp.	----		VO	----	83
Aphantopus hyperanthus	----		VO	----	83
Yphthimoides castrensis	Clicking	49	----	----	49
Charaxinae					
Prepona sp.	Grating	20	VO	----	55
Charaxes sp.	Grating	20,70	VO	----	41,55
Heliconiinae					
Heliconius erato	Clicking	32	HwM	Yes (P)	77
Heliconius cydno alithea	Clicking	32	----	----	
Papilionidae					
Parnassiinae					
Parnassius mnemosyne	Scratching,Rustling	4,35	----	----	
Hesperoidea					
Hesperidae					
Megathyminae					
Megathymus sp.	Clicking	71	----	----	
Hedyloidea					
Hedylidae					
Macrosoma heliconiaria	----		TO	Yes (B)	68,96

[1]In these cases, the proposed structure was a Vogel's organ when 'VO' is indicated, hindwing membrane for 'HwM', and a tympanal organ when 'TO' is indicated.

[2]Sound production was noted for these species, but the sound was not described.

Butterflies belonging to the subfamily Satyrinae (Nymphalidae) are distributed worldwide and are commonly referred to as satyrs and wood-nymphs, named for their typical woodland habitats. Generally, species of this subfamily exhibit some degree of swelling at the base of the forewing veins (Ackery *et al.*, 1999). There is also substantial morphological evidence suggesting that Vogel's organ is widely distributed throughout this group based on the findings presented here and other reports (Vogel, 1912; LeCerf, 1926; Bourgogne, 1951; Frings & Frings, 1956; Otero, 1990a; Ribaric & Gogala, 1996; Rydell *et al.*, 2003). To date, physiological investigation of hearing in this group is completely lacking and there is an outstanding need to examine a sense of hearing in Satyrinae. Here, in this thesis, this will be achieved by looking at a representative species, *Pararge aegeria*, the speckled wood butterfly, and then Satyrinae in general.

The primary objective of this thesis is to test for the possibility of hearing in a typical Satyrinae butterfly, using *Pararge aegeria* (Fig. 4). *P. aegeria* is widely distributed throughout Europe, Asia, and Northern Africa. This diurnal butterfly typically resides in or on the edge of woodland habitats (Goddard, 1962). There have been close to 100 publications in the last 2 decades featuring this species, relating to its unique life history traits and intriguing behaviour. *P. aegeria* is probably most well known for providing one of the first accounts of territorial behaviour in a butterfly. Males compete for territories of sunlit spots on the forest floor where they secure receptive females. Defense of these territories is achieved through aerial interactions in the form of spiral flight battles and horizontal chases (Davies, 1978). Most research on *P. aegeria* has focused on factors affecting which male will gain ownership of a given territory (Davies, 1978; Austad *et al.*, 1979), phenotypic (Shreeve, 1987; Van Dyck *et al.*,

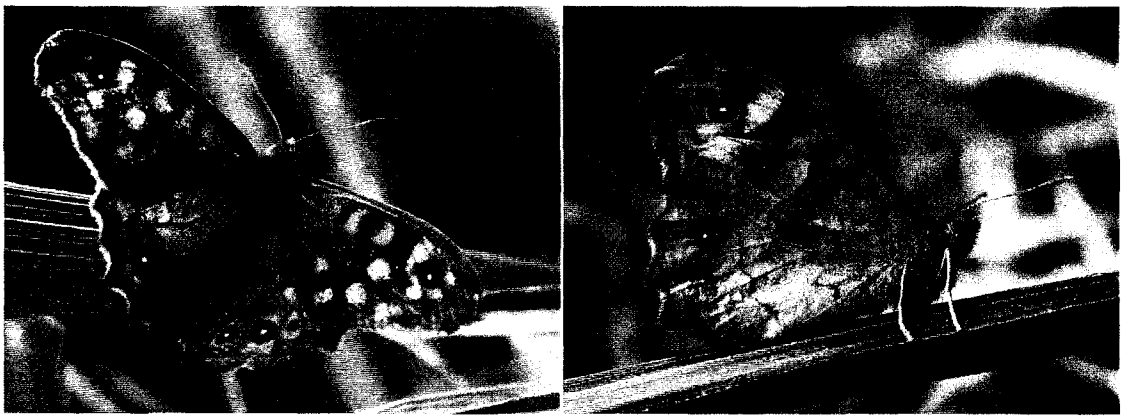

Fig. 4. The speckled wood butterfly, *Pararge aegeria*. (A) Live specimen in basking posture. (B) Live specimen in typical resting posture. Photo credit J. Yack.

1997a; Windig & Nylin, 1999) and environmental (Wickman & Wiklund, 1983; Shreeve, 1984; Dennis, 1986; Van Dyck *et al.*, 1997b) factors affecting male behavioural strategies, and the benefits of sunspot ownership (Stutt & Wilmer, 1998; Gotthard *et al.*, 1999). To date, there is no evidence for sound production or hearing in *P. aegeria.* Nevertheless, my preliminary observations revealed that they possess an externally prominent Vogel's organ. Despite the popularity of *P. aegeria* as a study subject, their potential sense of hearing has yet to be addressed. It is possible that this Vogel's organ functions in communication, the detection of bats, the detection of birds, or does not function in hearing at all. Hearing in *P. aegeria* will be studied by exploring the structure and function of the Vogel's organ. This will involve (1) describing the external morphology and innervation of the Vogel's organ, (2) recording physiological responses of nerve branches directly innervating Vogel's organ across a wide range of frequencies, and (3) conducting behavioural tests to determine the function of Vogel's organ.

The second objective of this thesis is to appraise Vogel's organ in the Satyrinae from a broader perspective. The subfamily Satyrinae is of particular interest as Vogel's organ has been reported in several species. The goals for this portion of the study are to (1) gain insight into how widespread this character is within the different clades of Satyrinae, (2) look for morphological variation that may have functional implications, and (3) test for a physiological response in a close relative of *P. aegeria*, *Lasiommata megera* and a local Satyrinae, *Cercyonis pegala.*

Results of this thesis will be discussed in terms of the function of hearing in *P. aegeria*, the significance of Vogel's organ in Satyrinae in general, and implications of this study with regards to the evolution of hearing in butterflies.

2. Materials and Methods

2.1 Animals

Dried specimens for both the *Pararge* and comparative components of this study were used for the purpose of external measurement, initial morphological investigation, and comparative study. *P. aegeria* were obtained from Melanie Gibbs from the University of Manchester (UK) and Dr. Hans Van Dyck of the Catholic University of Louvain (Belguim). Table 2 lists the sources of all Satyrinae (and Nymphalidae outgroup) specimens used for the comparative study. Complete collection information for the specimens is held in the Yack lab.

Live specimens of *P. aegeria*, *L. megera*, and *C. pegala* were used for the study of neural anatomy, physiology, and behaviour. *P. aegeria* pupae were purchased from Worldwide Butterflies (WWB) (UK) and obtained from Dr. Christer Wiklund of Stockholm University (Sweden). *Lasiommata megera* pupae were also purchased from WWB. *Cercyonis pegala* were collected from the wild along Dwyer Hill Road in Ottawa, ON, Canada.

P. aegeria and *L. megera* pupae were suspended with adhesive and cotton wool in a netted flight cage located indoors at room temperature with exposure to natural sunlight (at least 8 hours a day). The pupae were misted several times daily and the cage was covered in plastic to increase humidity. Upon eclosion, adults were kept in the flight cage with a tray of wheatgrass (*Triticum* sp.) lining the bottom. Cotton wool soaked in 10-30% sucrose solution was supplied in a plastic dish nested in the grass for the butterflies to feed on.

Table 2. Specimens (dried and live) used for comparative study and their sources.

Species	**Source**
Cercyonis pegala	Wild, collected from Ottawa region, ON, Canada
Satyrodes eurydice	Wild, collected from Ottawa region, ON, Canada
Aphantopus sp.	CNC (Ottawa, ON, Canada)
Charaxes sp.	CNC (Ottawa, ON, Canada)
Cithaerias sp.	CNC (Ottawa, ON, Canada)
Coenonympha pamphilus	CNC (Ottawa, ON, Canada)
Hipparchia semele	CNC (Ottawa, ON, Canada)
Heteronympha sp.	CNC (Ottawa, ON, Canada)
Maniola jurtina	CNC (Ottawa, ON, Canada)
Pierella sp.	CNC (Ottawa, ON, Canada)
Oeneis melissa	CNC (Ottawa, ON, Canada)
Brintesia circe	NMNH-SI (Washington, DC, USA)
Gnophodes parmeno	NMNH-SI (Washington, DC, USA)
Satyrus semele	NMNH-SI (Washington, DC, USA)
Stichopthalma howqua	NMNH-SI (Washington, DC, USA)
Gnophodes chelys	FMNH (Gainesville, FL, USA)
Manataria maculata	FMNH (Gainesville, FL, USA)
Stichopthalma howqua	FMNH (Gainesville, FL, USA)
Caligo eurilochus	LPS (Oxford, England)
Heliconius hecale	LPS (Oxford, England)
Idea leuconoe	LPS (Oxford, England)
Melanitis leda	Yack lab collection
Pararge aegeria	WWB, C. Wiklund, M. Gibbs, H. Van Dyck
Lasiommata megera	WWB
Elymnias hypermnestra	Unknown
Enodia anthedon	Unknown
Erebia sp.	Unknown
Pedaliodes phanias	Unknown

CNC = Canadian National Collection of Insects and Arthropods

FMNH = Florida Museum of Natural History, McGuire Center for Lepidoptera & Biodiversity

LPS = London Pupae Supplies

NMNH-SI = National Museum of Natural History, Smithsonian Institution

2.2 Morphology

2.2a *Pararge aegeria*

2.2a.i *External* – The main objectives of the morphological portion of this study were (1) to characterize and measure external characteristics of the Vogel's organ and associated structures of *P. aegeria* and (2) to compare dimensions between male and female *P. aegeria*, exploring the possibility of sexual dimorphism in Vogel's organ.

Dried male and female *P. aegeria* were used for external measurements. Measurements taken included the length, width, and surface area of both the inner and outer membranes of Vogel's organ, forewing length, and width of the subcostal (Sc), cubital (Cu) and anal (An) forewing veins. Scales were removed from veins and surrounding the Vogel's organ prior to measurement. Vogel's organ and surrounding cuticle were dissected out of the forewing following vein and forewing measurements. Photographs were taken using an Olympus SZX12 stereo microscope with a Zeiss Axiocam MRc5 camera, and measurements were made using Axiovision AC v. 4.1 software. The region of greatest width or length was measured. Two measurements were taken for each variable. A third measurement was obtained if the difference between the first two measurements was greater than 5%.

Average measurements and standard deviation of each variable was calculated for each specimen. Unpaired student's t-tests with 2-tailed distribution were used to assess differences between sexes. P-values of <0.05 were deemed significant. Body size dimorphism was assessed using forewing length measurements. In addition, 5 males and 5 females were qualitatively examined for sexually dimorphic differences in structure.

Selected air-dried specimens were prepared for scanning electron microscopy (SEM). Scales were removed from the forewing base, and Vogel's organ was cut away from the wing base. Preparations were sputter-coated with gold/palladium and examined using a JEOL JSM-6400 scanning-electron microscope.

2.2a.ii *Neural* – Determining the nature of innervation of the Vogel's organ was necessary for selecting a nerve branch to perform electrophysiological recordings from and to compare innervation patterns in other species, such as other Nymphalidae and Hedylidae, to assess possible homology. The terminology of Vogel (1912) was followed for peripheral projections of the nerve IIN1c. Description of the forewing base structures follows Sharplin (1963).

The thoracic ganglia of 5 live specimens and 20 specimens fixed in C&C (Chauthani & Callahan, 1966) were exposed through dorsal dissection of the thorax. Specimens, with heads removed, were pinned dorsal side up in a Petri dish lined with Sylgard. The pterothoracic ganglion (fused mesothoracic, metathoracic, and abdominal ganglia) and associated nerves were stained with 0.02% Janus Green B (Sigma Chemical Co.) (Yack, 1993), applied for 5-60 sec then decanted and replaced with saline (live specimens) or distilled water (fixed specimens). The distribution of nerves of the thoracic ganglia to their peripheral innervation sites, including Vogel's organ, were described and drawn using a camera lucida.

2.2b <u>Comparative Survey</u>

Representative Satyrinae and Nymphalidae outgroup species included in the comparative survey were chosen based on availability, and the phylogeny of Satyrinae by

Pena *et al.* (2006) (Fig. 5). Attempts were made to select representatives from each different clade of Satyrinae, including representatives from 7 of the 8 currently recognized tribes (Zetherini, Elymniini, Melanitini, Haeterini, Satyrini, Amathusiini, Brassolini, Morphini) and 6 of 11 Satyrini subtribes (Parargina, Lethina, Coenonymphina, Melanargiina, Manioliina, Pronophilina, Erebiina, Satyrina), following Pena *et al* (2006). No specimens were obtained for the tribe Zetherini or Satyrini subtribes Mycalesina, Euptychiina, and Ypthimina. Representatives from three other Nymphalidae subfamilies (Danainae, Heliconiinae, Charaxinae) were included as outgroups to Satryinae. Taxonomic placement of all species used in this study follows Pena *et al.* (2006) and dubious species were clarified by Niklas Wahlberg (pers. comm., Stockholm University, Sweden).

The forewing base of each specimen was photographed and measurements were taken of the forewing length and width of the forewing veins (Cu, Sc, An). The length of the forewing was measured as an indication of body size. The region of maximum and minimum width of the forewing veins (Cu, Sc, An) was measured and used in a ratio for all of the specimens to quantitatively assess the degree of vein inflation.

The presence or absence of Vogel's organ was then noted. In cases where Vogel's organ was present, the forewing of the specimen was removed and photographed. Measurements of Vogel's organ were taken following the same protocol as *P. aegeria.* Data from the forewing and Vogel's organ dimensions were used to examine a possible correlation between Vogel's organ and body size, using simple linear regressions.

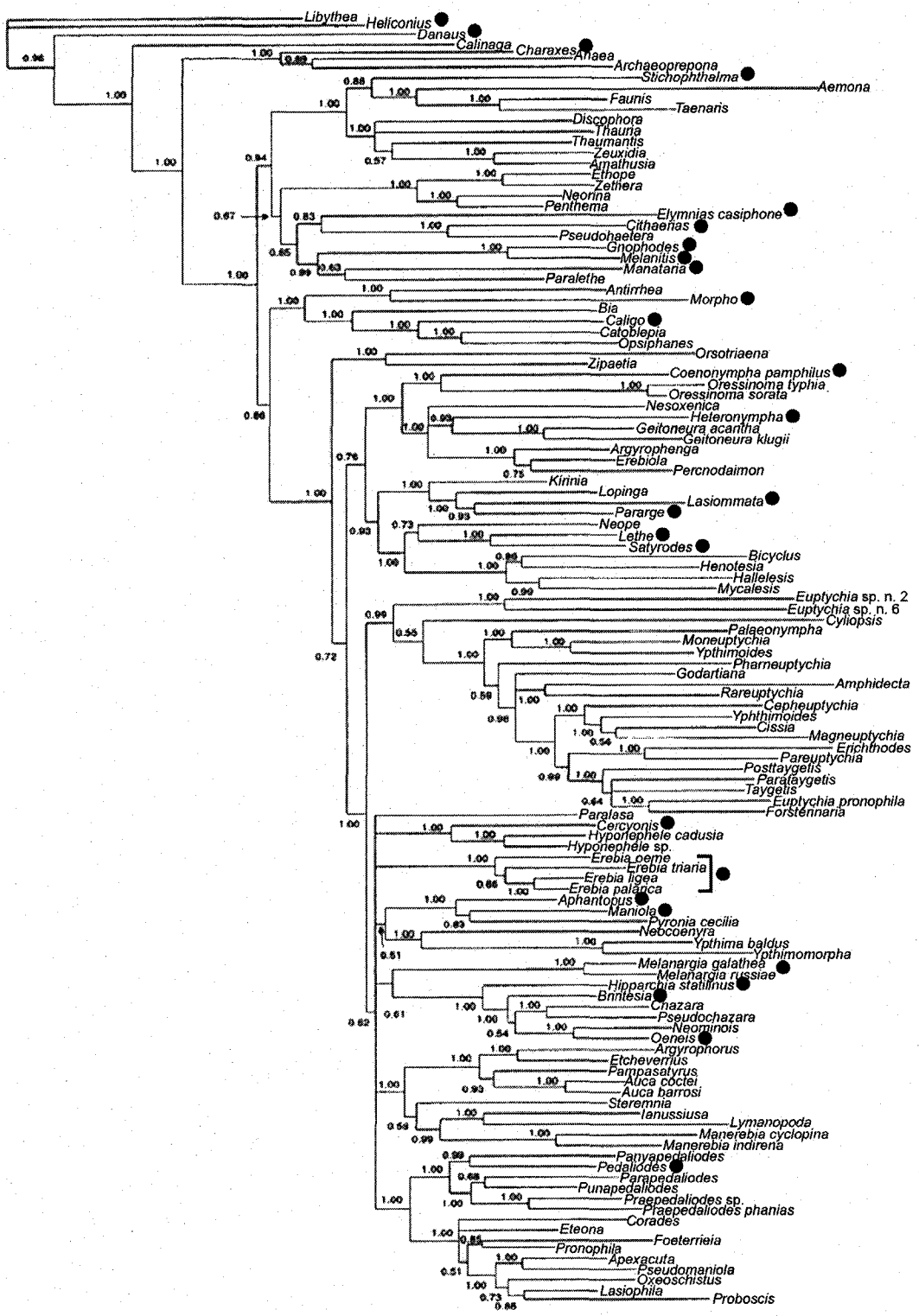

Fig. 5. Phylogenetic hypothesis for Satyrinae based on Bayesian analysis of three genes (*EF-1α*, *wg1*, and COI). Genera used in my comparative study are denoted with filled circles. Note that *Idea* was used in the place of *Danaus*; *Pierella* was added as a sister group to *Cithaerias*; *Lethe* was replaced with *Enodia*; *Satyrus* was added as a sister group to *Oeneis*. Replacements or additions follow Pena *et al.* (2006). Reproduced from Pena *et al.* (2006). For tribe placement, refer to Fig. 18.

Qualitatively, three different characters of Vogel's organ and related structures were assessed for each species. The first character was the absence or presence of a Vogel's organ. The second character, when a Vogel's organ was present, addressed the state of development of the Vogel's organ. Upon examination of all of the specimens, states of development were divided into three categories including poorly developed, moderately developed, and well developed. Please refer to the results section for a full description of these categories. The third character dealt with the degree of inflation of the Sc, Cu, and An veins at the base of the forewing. Inflated veins were deemed 'present' in cases where at least two of the three veins were inflated. Ratios of the narrowest and widest portion of a vein were determined for a quantitative measure of the degree of inflation of the forewing veins. Ratio values of 2.0 or higher indicated the presence of vein inflation. These characters were mapped onto a phylogenetic hypothesis for Satyrinae based on combined data of three genes (*EF-1α*, *wg1*, and COI), adapted from Pena *et al.* (2006), to evaluate if any phylogenetic patterns occur with regards to Vogel's organ.

2.3 Physiology

2.3a ***Pararge aegeria***

Live *P. aegeria* were positioned dorsal side up on a piece of modeling clay (Fig. 6) with a tunnel formed for sound transmission from the speaker to the ventral base of the forewing. The forewing and hindwing were uncoupled by placing the hindwing over top of the forewing such that the Vogel's organ was not obstructed. Dorsal dissection by removal of the tegula and underlying soft cuticle provided access to the recording site

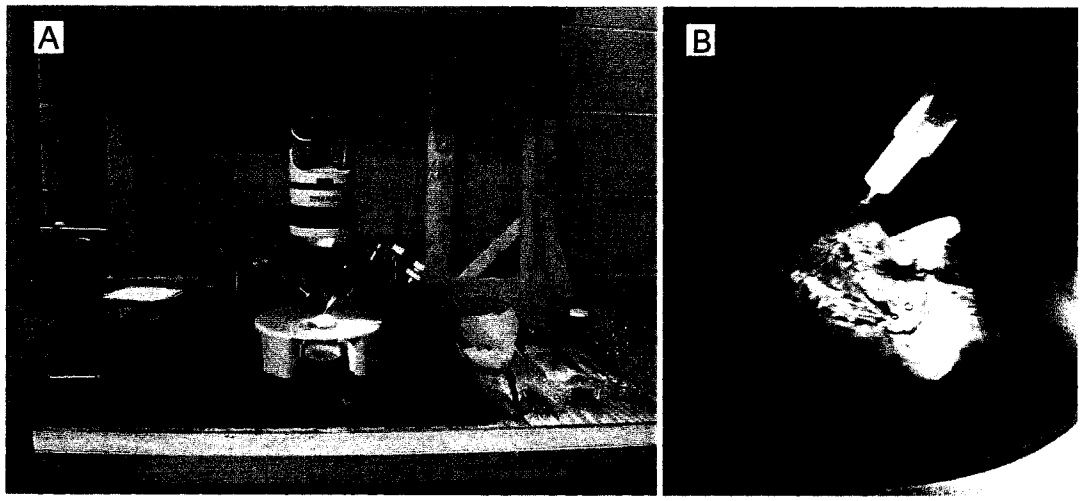

Fig. 6. Physiology set-up for extracellular recordings. (A) Custom-built physiology rig. (B) *P. aegeria* mounted for recordings dorsal side up on a modeling clay base. The recording electrode is fashioned within an empty syringe, and positioned towards the recording site using a micro-manipulator.

(where IIN1c trifurcates). In total, recordings using 28 animals (13 female, 6 male, 9 sex unknown) were attempted. In the end, 9 females and 1 male *P. aegeria* recordings were used to construct audiograms. Vogel's organs were inspected following recordings to ensure that they were intact and in good condition.

Extracellular recordings were performed by isolating the middle (NII) and/or posterior branch (NIII) of the IIN1c nerve on a sharpened stainless steel hook electrode, while a reference electrode was inserted into the abdomen. Petroleum jelly was injected under the hooked nerve to prevent dessication and to isolate electrical activity of the electrode from the haemolymph of the animal. Neural activity was amplified using a Grass P15 amplifier, with output displayed on a Tektronik THS720A oscilloscope.

Stimulus presentation was achieved using a Tucker Davis Technologies (TDT) RX-6 multifunction processor. Test frequencies in the range of 500 Hz – 4 kHz were presented using a TDT SA1 Power Amp powering a generic 6-inch woofer. Test frequencies in the range of 3 kHz to 25 kHz were generated using a TDT PA5 programmable attenuator coupled with a Motorola KSN 1078A 2-inch cone tweeter. Speakers were positioned approximately 50 cm from the animal. Shaped stimulus pulses were presented with 30 ms sustained/5 ms rise-fall duration at intervals of 1 pulse per second. Sound intensities were determined using a B&K Type 2239 sound level meter positioned 50 cm from the speaker for each frequency tested after each audiogram was determined. The sound level meter was routinely calibrated using a B&K sound level calibrator (Type 4231).

Audiograms (plot of stimulus frequency vs. intensity) were constructed from the electrophysiology data in order to determine the range of frequencies and intensities for

which the auditory neurons within IIN1c nerve branch are selective. For each frequency presented, the threshold was determined using the 'just noticeable difference' method by gradually increasing the broadcast intensity of the stimulus until audible nerve activity synchronized with the stimulus pulses was elicited (following Yack *et al.*, 2000). Median thresholds were calculated for each frequency. The median audiogram and best frequency were determined using frequencies for which two or more thresholds were determined ($n \geq 2$). The Q_{10} value, a measure of the tuning curve width, was calculated from the audiograms by dividing the best frequency by the frequency range 10 dB above threshold.

For offline analysis, recordings of neural responses were digitally captured on a Fostex FR-2 Field Memory Recorder and then played in Sony Sound Forge 8.0 where latencies were determined. Latencies were established from the onset of the stimulus pulse to the first sensory spike and then first motor spike, not taking into account the time it takes for the stimulus to reach the sound receptor of the animal. Latencies were also measured from compound action potentials that were obtained by triggering the oscilloscope with the sound stimulus and time-averaging 16 sweeps consecutively.

Note: In an attempt to improve the signal-to-noise ratio, the use of a double hook electrode was employed (Fig. 7). However, this did not yield an improvement in the signal:noise ratio.

2.3b *Cercyonis pegala* and *Lasiommata megera*

For comparative purposes, recordings were performed using *Cercyonis pegala* (Satyrini, Satyrinae) and *Lasiommata megera* (Satyrini, Satyrinae) following the

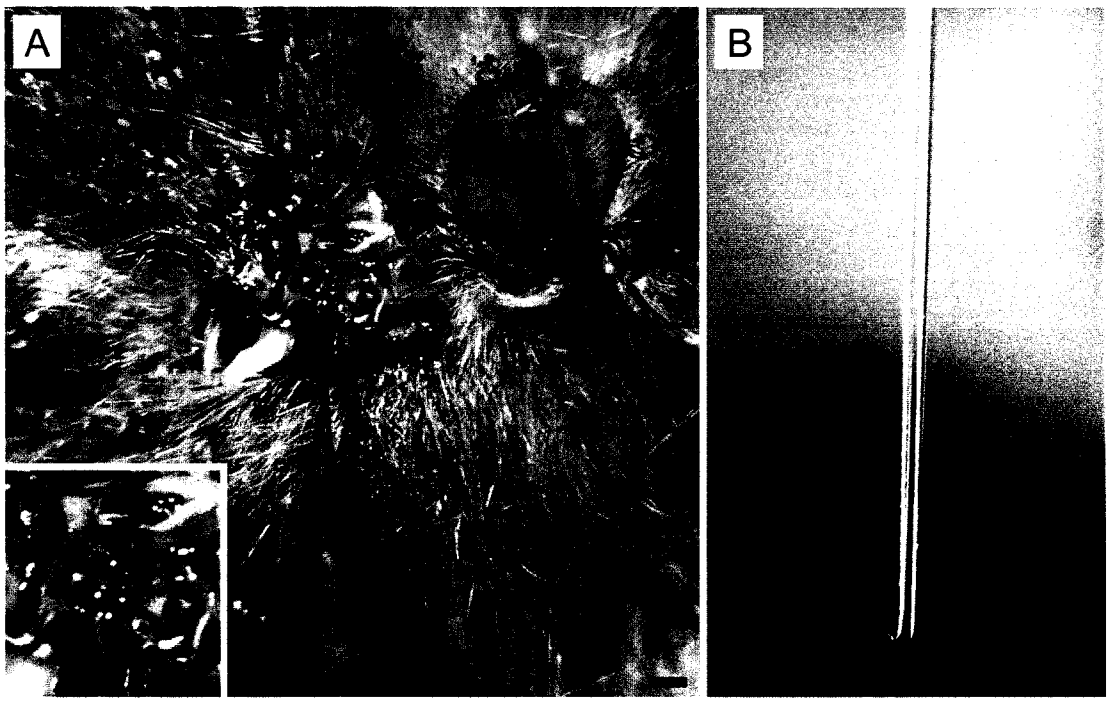

Fig. 7. Double-hook electrodes used in physiological recordings in an attempt to increase the signal:noise ratio. (A) Recording site under the tegula with the double electrode hooked onto IIN1c. Inset: a closer view of the hooks under IIN1c. (B) Close-up view of the double-hook electrode. Scale bars, 1mm.

same protocol as outlined for *P. aegeria*. In total, recordings were attempted on 6 *C. pegala*, resulting in a successful recording from 1 individual (sex unknown). For *L. megera*, recordings were attempted on a total of 7 different individuals, for which audiograms were constructed from 3 individuals (1 male, others sex unknown). Comparative physiology methodology differed from *P. aegeria* in that intensity series were also conducted for *L. megera*. After the best frequency was determined to be 3 kHz, the animal was presented with 10 stimulus pulses at increasing intensity level starting at 6 dB below threshold and increasing at intervals of +6 dB up to a maximum intensity of +36 dB above threshold. Recordings of neural responses at each intensity level were digitally captured using a Fostex FR-2 Field Memory Recorder then played in Sony Sound Forge for subsequent analysis.

2.4 Behaviour

P. aegeria were tested for behavioural responses to both low and high frequency sound stimuli while tethered (at rest) and in-flight.

2.4a *Behavioural Response to Sound*

2.4a.i Tethered - A total of 10 (6 males, 4 females) were tested for a behavioural response to both low- and high-frequency sounds at rest. The legs of *P. aegeria* were removed and the butterflies were tethered on the ventral surface to a wooden stick using hair-removal wax. Using a clamp and stand, the butterflies were positioned approximately 40 cm from the speaker within a Faraday cage (0.5 m x 0.5 m x 0.5 m).

Trials were recorded using a Sony DCR-HC85 NTSC digital video camera, where recording was initiated just prior to stimulus presentation and continued for 10 sec

following the last stimulus pulse. Butterflies were stimulated with 3 pulses (1 sec duration) at intervals of 10 sec. Presentation of the sound stimuli was achieved using a TDT RX-6 Multifunction processor. Each butterfly was subjected to stimuli in the range of 0.5 kHz to 30 kHz, with 120 sec breaks observed between frequencies. Sound stimuli in the range of 500 Hz to 3 kHz were presented using a TDT SA1 Power Amp and a generic 6-inch woofer. Sounds in the range of 3 kHz – 30 kHz were presented using a TDT PA5 programmable attenuator and a Motorola KSN 1078A 2-inch cone tweeter. For sounds >19 kHz, an Ultra Sound Advice Mini-2 Bat Detector (Summit, Birmingham, England) was used to provide an indication of ultrasonic response. Following each trial, a B&K Type 2239 sound level meter was used to measure sounds intensities of each frequency tested at a distance of 40 cm. Intensity measurements for frequencies >18 kHz are considered rough estimates due to limitations of the sound level meter. In addition, an electronic dog whistle (Pet Trainer™) was used to test for tethered responses to intense ultrasound (26 kHz +). These trials were preliminary and sound intensities were not measured due to equipment limitations. However, other studies using the dog whistle cite intensities of about 110 dB SPL at 1 m (Rydell *et al.*, 2003).

For analysis, behavioural responses were classified as any visible motor movement (i.e. proboscis extension, slight twitching of closed or open wings, forward movement of wings, partial or total wing opening, or fluttering of wings) occurring within a 2 sec latency to sound stimuli.

2.4a.ii In-flight – Trials were performed on 5 free-flying *P. aegeria* (sex unknown). A white canvas drop cloth (10' x 11') was set up against a wall in a room with a temperature of approximately 23°C. Distance references were indicated on the

backdrop and floor for quantification of behavioural responses. Behavioural observations were recorded using a digital video recorder Sony Digital 8 DCR-TR7000 NTSC video camera set up on a tripod positioned 4.5 m away from the wall. A visual reference (waving hand) to the onset of ultrasound stimulation was used to provide an indication of when the stimulation occurred in the recorded video. Each butterfly was released at the side of the sheet. *P. aegeria* were exposed to sound stimuli using an electronic dog whistle (Pet Trainer™), triggered manually. As soon as the butterfly flew into camera view, it was presented with 3 consecutive ultrasonic pulses, each 1 sec in duration, at intervals of 5 sec at distances of 1.5 m, 3 m, and 5 m. The behaviour of the butterfly was recorded for 90 sec following exposure to ultrasound. Each individual butterfly was used only once to prevent habituation effects. Video tapes were then analyzed for any indication of a behavioural response, which was defined as any interruption of flight or increase in flight speed occurring within 2 sec of the stimulus presentation.

Preliminary observations were also made for testing for an in-flight behavioural response to low-frequency sound stimuli (n= 3). *P. aegeria* were held in a flight cage with a speaker positioned at a distance of ~40 cm. Stimulus presentation and parameters followed the same protocol as the tethered trials. The butterflies were stimulated with low-frequency sounds (0.5 kHz – 25 kHz) as they flew about the cage. All behaviour (pre- and post- stimulus) was recorded using a Sony Digital 8 DCR-TR7000 NTSC video camera. The videos were then analyzed for any response occurring within 2 sec of the stimulus presentation.

2.4b *Sound Production*

Preliminary observations were made to test the possibility of sound production in

P. aegeria. Free-flying butterflies were monitored with an Ultra Sound Advice Mini-2 Bat Detector and any detection of ultrasound was noted. To test for low-frequency sound production, butterflies were released into the greenhouse and were allowed to fly freely. Any audible sound being produced was noted. Since there was no indication of sound production, formal trials were not carried out.

2.5 Bird Flight Recordings

Bird flight sounds were characterized through recordings of black-capped chickadees (*Parus atricapillus*) and grackles (*Quiscalus quiscula*). Recordings were obtained at two different locations. Preliminary recordings were performed using chickadees in the field at Stoney Swamp conservation area (Ottawa, ON, Canada). Chickadees flew up to handfuls of seed where a Sony ECM-MS908C microphone (frequency range: 100 Hz – 15 kHz) was held at a distance of 5-10 cm coupled with a Sony DCR-TRV19 NTSC digital video camera. Intensity data were not obtained for these recordings, but frequencies were determined from spectrograms created in Canary 1.2.4 bioacoustics software program (Cornell Bioacoustics Research Program, Ithaca, NY, USA).

Recordings of chickadees and grackles were also obtained at a private residence in Chelsea, Quebec, Canada. All flights to a feeder were captured on video using a Sony DCR-HC85 NTSC digital video camera. A Sony ECM-MS957 microphone (frequency response up to 18 kHz) was set up on a tripod approximately 10 cm under a well-established feeder. Flight sounds were captured using a Sony DAT PCM-M1 digital

audio recorder at a sampling rate of 48 kHz, for which the frequency response falls off at 20 kHz.

Flight sounds were digitized and then saved onto a computer where they were analyzed using Raven 1.3 sound analysis software (Cornell Bioacousics Research Program, Ithaca, NY, USA). Waveforms for flight sounds were created and used to construct power spectra where the fast Fourier transformation (FFT) window (Hanning) used for computing the spectra was 512 points.

Sound intensities for flight sounds were determined by comparing the intensities to a calibrated system. Pure tone sounds at various frequencies and distances were delivered to the DAT under the same recording levels and at the same distance used for the flight recordings. The sounds were then output onto a Tektronik THS720A oscilloscope where peak-to-peak amplitude was measured. For each frequency at a given distance, a range of intensities were measured. The DAT recordings of flight sounds were then compared to the calibrated sounds (using the resultant calibration curves), and intensities were determined.

3. Results

3.1 Morphology

3.1a *Pararge aegeria*

3.1a.i External - The Vogel's organ lies on the ventral surface of the forewing at the base of the cubital vein (Figs. 8A, 9A, 12C). The organ comprises a thinned membrane set within the cubital vein with cuticular borders provided by the cubital vein. When the wings are held up at rest, the shape of the hindwing margin is such that the

portion of the forewing housing the membrane is fully exposed. The membrane is protected by a fringe of coarse scales running along the longitudinal axis of the Vogel's organ, extending out and over the base of the subcostal vein. Finer scales extend from the subcostal vein region, bordering the fringe of coarse scales on either side (Figs. 8B, 9B).

Overall, the Vogel's organ is oval in shape and includes a distinct inner membrane that is also oval. The average length of the Vogel's organ is 676.36 ± 70.59 μm in males (n = 13) and 711.63 ± 49.56 μm in females (n = 13). The average width of the Vogel's organ is 383.30 ± 48.27 μm in males (n = 13) and 401.54 ± 33.59 μm in females (n = 13). Observed under the light microscope, the outer membrane appears translucent in live or fresh specimens, while the inner membrane is more transparent and provides a view of a whitish strand that appears to attach to the center of the inner membrane (Figs. 8C, 9C). Table 3 summarizes the dimensions of ear and wing length in both males and females. Sexual dimorphism was not apparent with respect to the lengths and widths of the inner and outer membranes. However, a significant difference was noted between sexes in the surface area of the outer membrane, with the female being slightly larger than the male. Since no significant differences were noted between the body size of male and females (Table 3), and there was no significant differences between length and width of the Vogel's organ, sexual dimorphism of the Vogel's organ is not proposed. Qualitative assessment of sexual dimorphism in Vogel's organ involved looking at the overall shape, surrounding scale density and patterns, structure of the cuticular borders, topography of the inner and outer membranes, and other general features. This assessment did not reveal any obvious sexual dimorphism of the Vogel's

Fig. 8. The external morphology of Vogel's organ in male *Parargo aegeria*. (A) A view of the ventral hindwing, forewing, and head (region of the tympanal organ is circled). (B) Closer view of Vogel's organ on the ventral surface of the forewing (with hindwing intact) in a live specimen. (C) Close up of Vogel's organ in a live specimen (arrow points to the attachment site). Hindwing and surrounding scales are removed. Scale bar, 100 µm.

Fig. 9. The external morphology of Vogel's organ in female *Pararge aegeria*. (A) A view of the ventral forewing and head (region of tympanal organ is circled), with the hindwing removed. Scale bar, 1mm. (B) Closer view of Vogel's organ on the ventral surface of the forewing (with hindwing intact). Arrow points to the anterior edge of the Vogel's organ (C) Close up of Vogel's organ in a dried specimen (arrow points to attachment site). Hindwing and surrounding scales are removed. Scale bar, 100 µm.

Table 3. External measurements of the Vogel's organ and wing length in *P. aegeria.*

	Length of Vogel's Organ (μm) (mean ± std. deviation)		Width of Vogel's Organ (μm) (mean ± std. deviation)		Area of Vogel's Organ (mm) (mean ± std. deviation)		Length of Forewing (cm) (mean ± std. deviation)
	Outer Membrane	Inner Membrane	Outer Membrane	Inner Membrane	Outer Membrane	Inner Membrane	(cm)
Male (n=13)	676.36 ± 70.59	383.30 ± 48.27	322.11 ± 28.67	200.74 ± 19.32	155.67 ± 20.27	55.80 ± 16.77	2.05 ± 0.18 (n=16)
Female (n=13)	711.63 ± 49.56	401.54 ± 33.59	329.53 ± 27.69	217.37 ± 41.93	172.96 ± 15.82	64.21 ± 8.01	2.13 ± 0.11 (n=16)
P-value	0.1534	0.2744	0.5086	0.2063	0.0255	0.1159	0.1373

organ. Correspondingly, there does not appear to be any significant difference in body size, determined from forewing length, between males and females (P value = 0.1687, n = 16 males, 16 females).

The SEMs reveal differences in surface topography of the inner and outer membranes of the Vogel's organ (Figs. 10A, 11A). The outer membrane appears to be covered in ripples (Figs. 10B, 11B), while the inner membrane is much smoother (Figs. 10C, 11C) and is covered in very small setae measuring about 0.8μm long (Figs. 10D, 11D).

The basal portions of the forewing An, Sc, and Cu veins are dilated more than twice the width of the more apical sections in both males and females. The greatest width of the veins, on average, are: 675.65 μm ± 59.32 (n = 28) for the Sc vein, 644.37 μm ± 105.03 (n = 30) for the cubital vein, and 245.43 μm ± 32.50 (n = 30) for the anal vein. These values represent data for both males and females combined. The average measurements of the Cu and An vein widths did not reveal a significant differences in size between sexes (Cu P-value = 0.3528; An P-value = 0.1393; n = 16 females, 14 males). However, females had a significantly wider Sc vein compared to males (P-value = 0.0141, n = 15 females, 13 males), where the female had greater width.

3.1a.ii Neural - The mesothoracic nerve IIN1 leaves the lateral edge of the pro-mesothoracic connectives (Fig. 12A). The largest branch of IIN1, IIN1c, extends laterally, under and around the dorsoventral flight musculature, passing beneath the tegular arm. As IIN1c passes under the tegular branch, a fine branch extends anteriorly towards the tegula, which it innervates. A trifurcation was observed immediately past the tegular branch and the tegular arm. Dorsal removal of the tegula and soft underlying

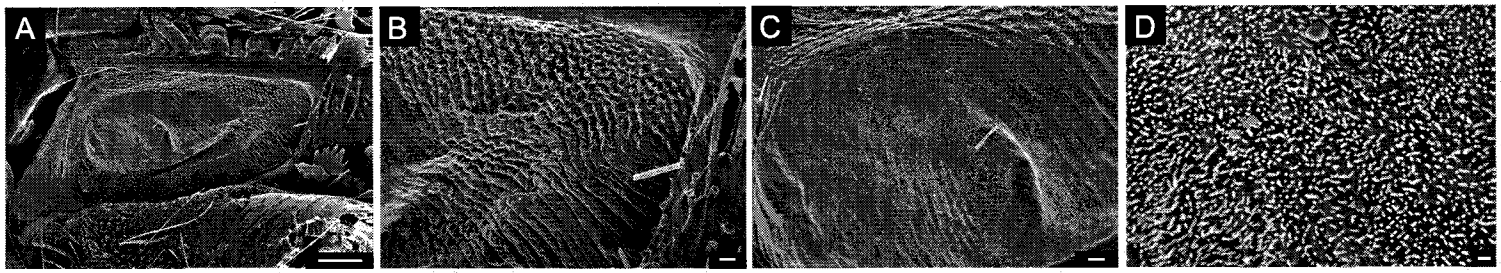

Fig. 10. Scanning electron micrograph of Vogel's organ of male *Pararge aegeria*. (A) View of the whole Vogel's organ and surrounding cuticular borders. Scale bar,100µm. (B) View of the distal edge of the outer tympanal membrane with ripples covering the surface. Scale bar,10µm. (C) Close-up view of the center of the inner membrane, focusing on the region of the attachment site. Scale bar,10µm. (D) Close-up of tiny setae covering the entire surface of the inner tympanal membrane. Scale bar,1µm.

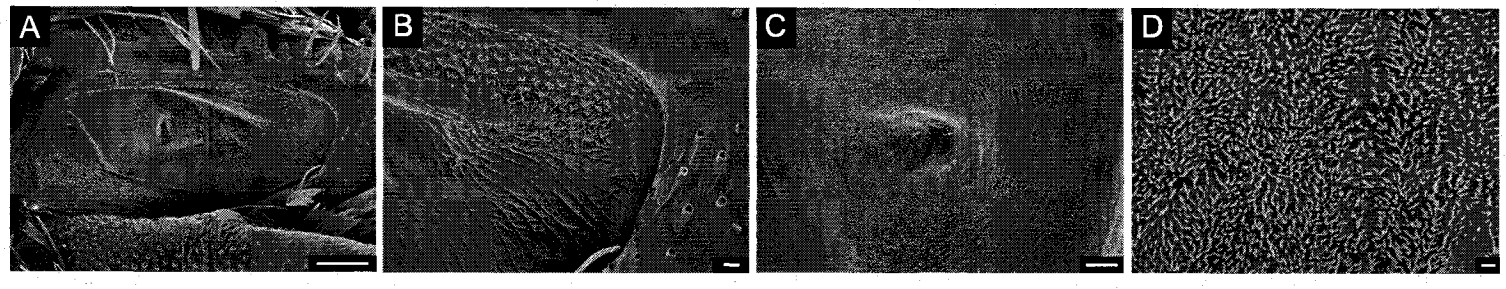

Fig. 11. Scanning electron micrograph of the Vogel's organ of female *Pararge aegeria*. (A) View of the whole Vogel's organ and surrounding cuticular borders. Scale bar, 100μm. (B) View of the distal edge of the outer tympanal membrane with ripples covering the surface. Scale bar, 10μm. (C) Close-up view of the center of the inner membrane, focusing on the region of the attachment site. Scale bar, 10μm. (D) Close-up of tiny setae covering the entire surface of the inner tympanal membrane. Scale bar, 1μm.

cuticle provides access to the trifurcation for physiology recordings (Fig. 12B). The anterior branch of IIN1c, NI, extends anteriorly in the direction of the subcostal vein (Fig. 12C). NI branches into two, where one branch follows the anterior margin of the forewing and the other extends up into the subcostal vein. NI does not innervate Vogel's organ. The middle branch, NII, branches into two before reaching the base of the radial vein. The more anterior of the two branches extends into the radial vein while the other branch leads to the very basal portion of the Vogel's organ where it forms a bulbous structure which connects *via* a prominent attachment strand to the centre of the inside of the tympanum of the Vogel's organ. The posterior branch of IIn1c, NIII, branches into two at the base of the forewing. The more anterior branch leads to the basal portion of the Vogel's organ where it connects to another bulbous structure which lies within the bottom portion of the Vogel's organ. The more posterior branch of NIII extends up along the margin between the anal vein and edge of Vogel's organ where it appears to innervate a third bulbous structure which lies along the margin of Vogel's organ.

Attempts were made to conduct histological work on Vogel's organ, however successful results were not obtained. Since histology could not be performed, it cannot be confirmed that these bulbous structures comprise chordotonal organs, however it is surmised.

3.1b Comparative Study

The forewing bases of 25 species representing 7 out of 8 currently recognized Satyrinae tribes were surveyed. Three species from representative outgroups of 3 Nymphalidae subfamilies were also included. Table 4 summarizes the presence or

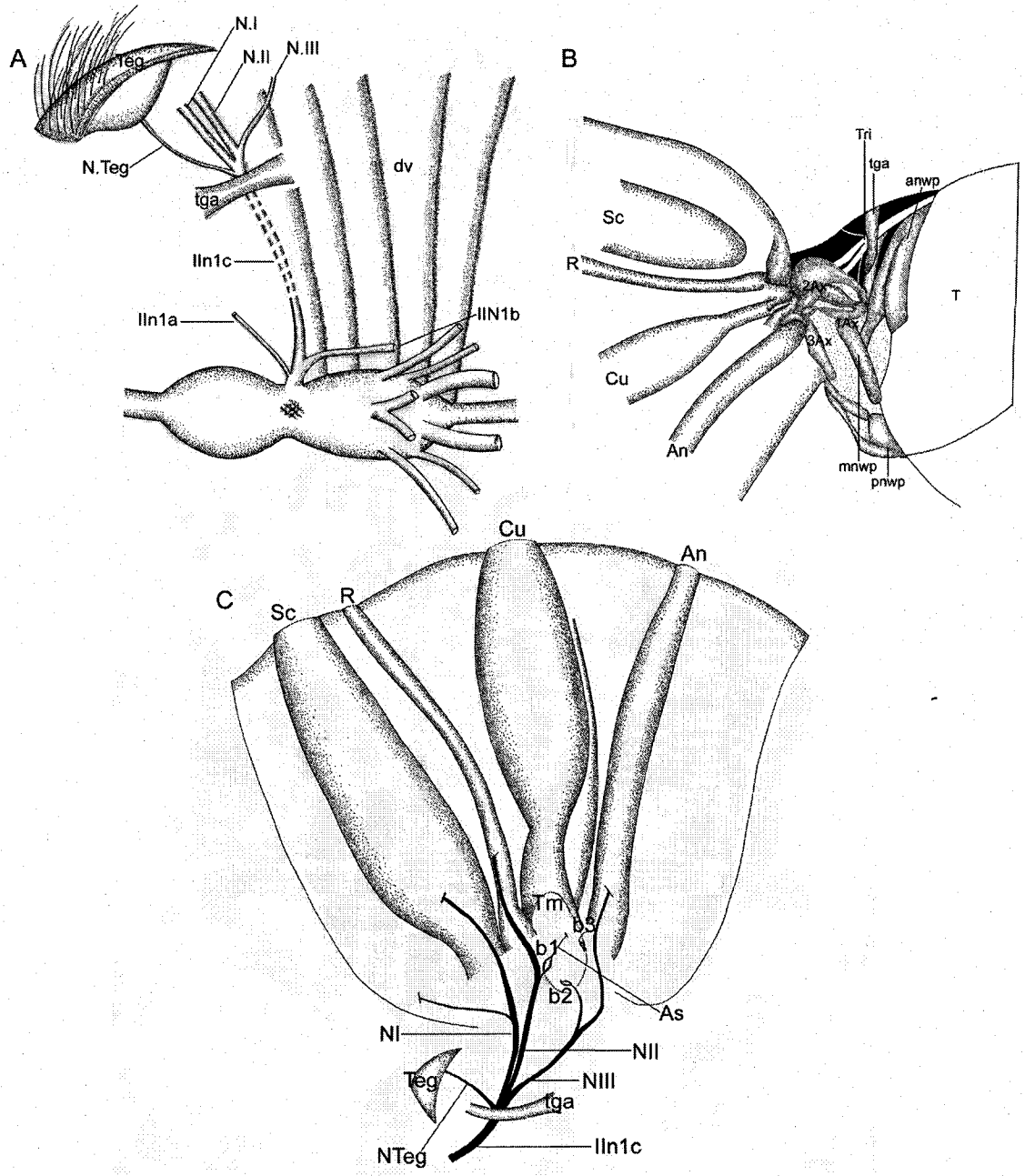

Fig. 12. Schematic diagrams of the mesothoracic wing nerve IIN1c and its peripheral projections in *Pararge aegeria*. (A) Dorsal view of the pterothoracic ganglion, depicting nerve root IIN1 with branches a, b, and c. Dashed line represents IIN1c passing beneath muscle. (B) Dorsal view of the left forewing base. Removal of the tegula and soft underlying cuticle provides access to the trifurcation of IIN1c (nerves shown in white). anwp, anterior notal wing process; Ax1, 1st axillary sclerite; Ax2, 2nd axillary sclerite; Ax3, 3rd axillary sclerite; C, costal vein; Cu cubital vein; mnwp, median notal wing process; pnwp, posterior notal wing process; R, radial vein; Sc, subcostal vein; T, thorax. (C) Ventral view of the left forewing and base. The trifurcation is shown with NI, NII, and NIII extending up the forewing, the location of their peripheral targets. NII connects to a bulbous structure (b1) *via* an attachment strand (As) to the tympanal membrane (Tm). NIII innervates two other bulbous structures (b2, b3).

Table 4. List of species used in the comparative study including taxonomic ranks (following Pena *et al.*, 2006) and presence or absence of Vogel's organ. Those Satyrinae marked with an asterisk (*) denote members of tribes that have just recently been added to the Satyrinae subfamily, and previously were grouped together in their own subfamily, Morphinae.

Satyrinae	**Vogel's Organ**
Elymniini	
Elymnias hypermnestra	Present
Zetherini	
No data available	
Melanitini	
Manataria maculata	Present
Gnophodes sp.	Present
Melanitis leda	Present
Haeterini	
Cithaerias sp.	Present
Pierella sp.	Present
Satyrini	
Parargina	
Lasiommata megera	Present
Pararge aegeria	Present
Lethina	
Enodia anthedon	Present
Satyrodes eurydice	Present
Mycalesina	
No data available	
Coenonymphina	
Coenonympha pamphilus	Present
Euptychiina	
No data available	
Hypocystina	
Heteronympha sp.	Present
Ypthimina	
No data available	
Melanargiina	
Melanargia sp.	Present
Maniolina	
Maniola jurtina	Present
Aphantopus sp.	Present
Pronophilina	
Pedaliodes phanias	Present
Erebiina	
Erebia sp.	Present
Satyrina	
Hipparchia semele	Present
Satyrus symele	Present
Brintesia circe	Present
Oeneis melisssa	Present
Subtribe unknown	
Cercyonis pegala	Present
Amathusiini*	
Stichophthalma howqua	Present

Brassolini*		
	Caligo eurilochus	Present
Morphini*		
	Morpho peleides	Present
Danainae		
Danaini		
	Idea leuconoe	Absent
Heliconiinae		
Heliconiini		
	Heliconius hecale	Absent
Charaxinae		
Charaxini		
	Charaxes sp.	Present

absence of Vogel's organ in each of these species, which are organized according to their current taxonomic groupings. Absence of Vogel's organ was defined by forewings with no modification of the wing base in terms of surface properties of the cuticle or reduction of scales. *Heliconius* (Heliconiinae) and *Idea* (Danainae) were the only two specimens found to be completely lacking a Vogel's organ (Fig. 13).

Varied degrees of Vogel's organ development were seen within Nymphalidae and the state of development was divided into three categories. The first category, poorly developed, is characterized by cases where a thinning of the cuticle at the base of the forewing is visible, typically identified by a difference in colouration and transparency of the cuticle. However, this region is not surrounded by a defined cuticular border and the overall shape of this area is not uniform. A poorly developed Vogel's organ was found in *Stichophthalma* (Fig. 14). The second category, moderately developed, includes Vogel's organs where a uniformly shaped area of thinned cuticle is present, but thick cuticular borders do not surround this area completely. *Charaxes* and *Caligo* were found to have Vogel's organs of moderate development (Fig. 15). The third category, well-developed, is defined by a region of very thin cuticle at the base of the forewing surrounded on all edges by a distinct border of cuticle. Well-defined Vogel's organs include a distinct inner membrane (Fig. 16). All of the Satyrinae were found to have a well-defined Vogel's organ, with the exception of *Stichophthalma* and *Caligo*. Well-defined Vogel's appear to be concentrated in the Satyrinae.

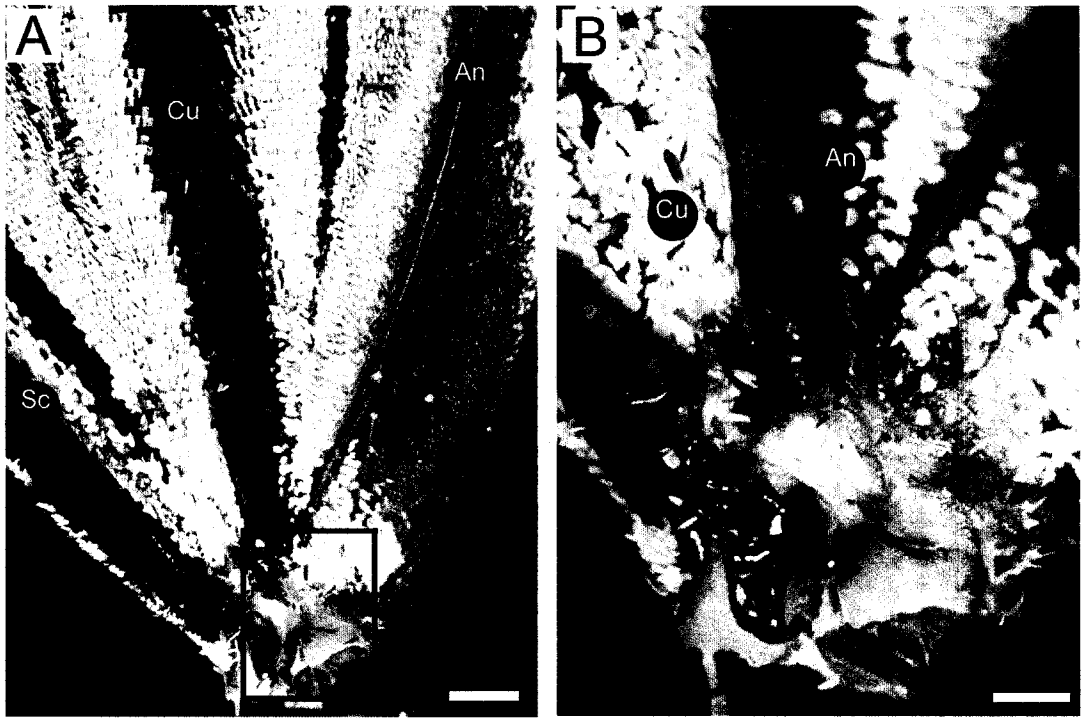

Fig. 13. Representative specimen exhibiting absence of the Vogel's organ. (A) Ventral aspect of the left forewing of *Idea* sp. (Danainae) with non-inflated forewing veins. Veins labeled as An, anal; Cu, cubital; Sc, subcostal. Scale bar, ~1.5mm. (B) Close-up view of the base of the forewing (area boxed in (A)), exhibiting absence of Vogel's organ. Scale bar, ~0.5mm.

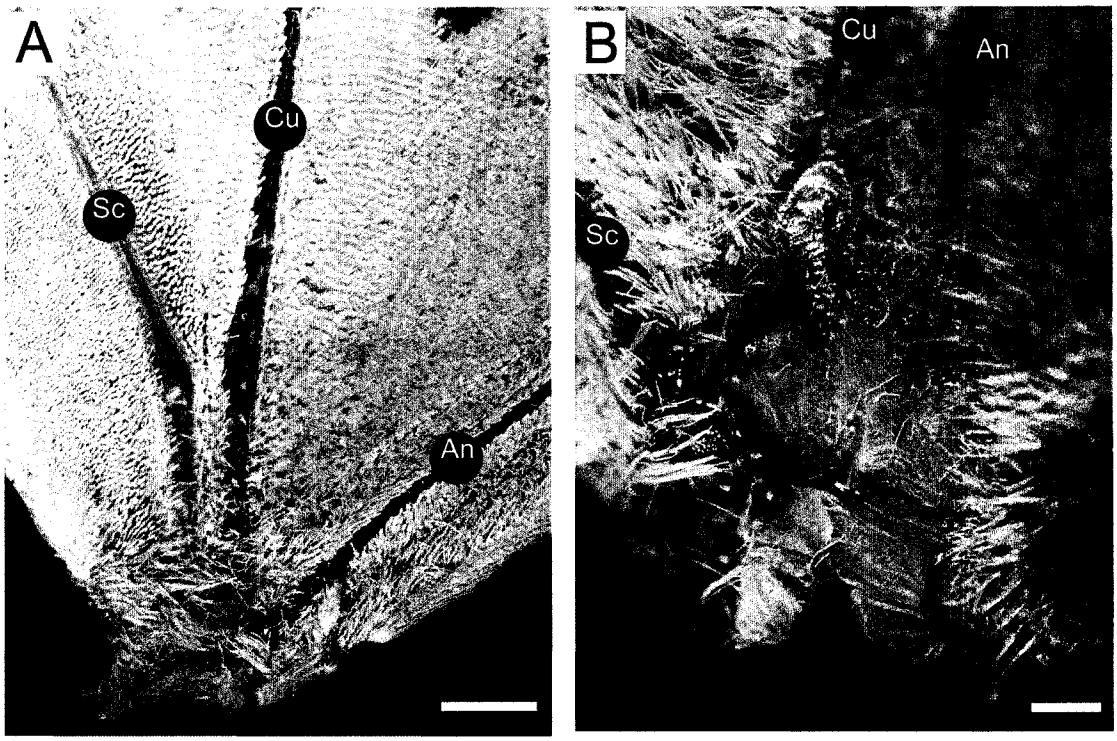

Fig. 14. Representative specimen exhibiting a poorly developed Vogel's organ. (A) Ventral aspect of the left forewing of *Stichophthalma* sp. (Amathusiini, Satyrinae) with non-inflated forewing veins. Veins labeled as An, anal; Cu, cubital; Sc, subcostal. Scale bar, ~1.5mm. (B) Close-up view of the base of the forewing with a poorly-developed Vogel's organ. Scale bar, ~0.5mm.

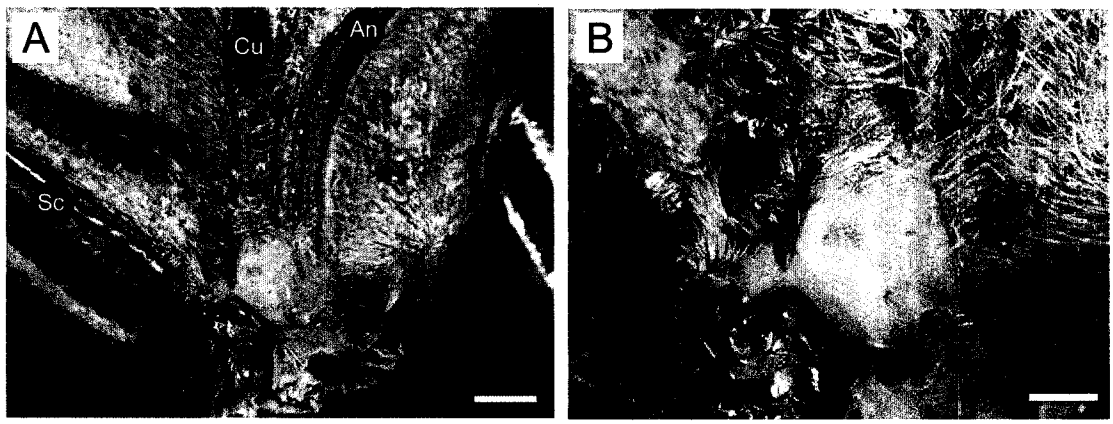

Fig. 15. Representative specimen exhibiting a moderately-developed Vogel's organ. (A) Ventral aspect of the left forewing of *Caligo* sp. (Brassolini, Satyrinae) with non-inflated forewing veins. Veins labeled as An, anal; Cu, cubital; Sc, subcotal. Scale bar, ~1mm. (B) Close-up view of the base of the forewing, exhibiting moderate development of the Vogel's organ. Scale bar, ~0.5mm.

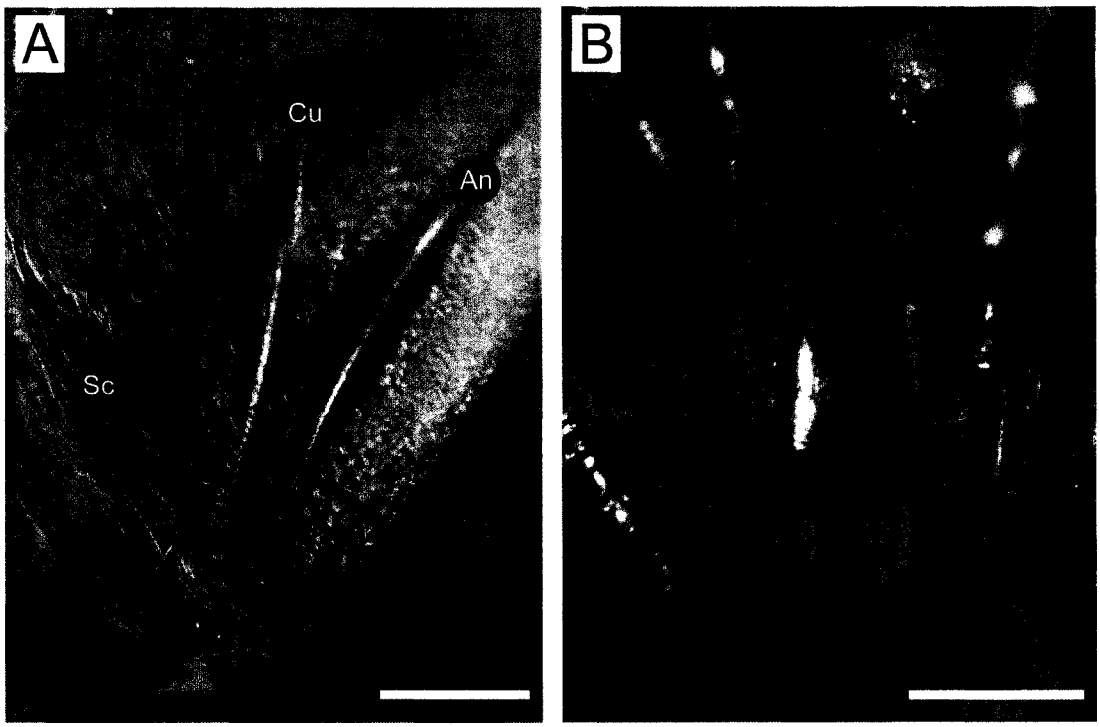

Fig. 16. Representative specimen exhibiting a well-developed Vogel's organ and inflated veins. (A) Ventral aspect of the right forewing of *Coenonympha* sp. (Satyrini, Satyrinae) with inflated forewing veins . Veins labeled as AN, anal; CU, cubital; SC, subcostal. Scale bar, ~1mm. (B) Close-up view of the base of the forewing with a well developed Vogel's organ. Scale bar, ~0.25mm.

The third character, inflation of the forewing veins, was found to be restricted to the Satyrinae (Fig. 17A), with a few exceptions. Inflation of forewing veins was defined when at least two of the forewing veins yielding a ratio (widest portion:narrowest portion) of 2.0 or higher, indicating inflation to the degree of twice the size of the narrowest portion of the vein. Satyrinae without inflated veins were *Stichophthalma*, *Morpho*, *Caligo*, *Gnophodes*, *Melanitis*, and *Manataria* (Fig. 17 B,C, D). Non-inflated vein specimens had ratio values of < 2.0 for at least two of the forewing veins. For example, *Idea* had ratio values of 1.27 for the Sc vein, 1.17 for the An vein, and 1.20 for the Cu vein. Whereas, *Coenonympha*, which exhibited a high degree of inflation, generated values of 3.86 for the SC vein, 3.5 for the AN vein, and 4.25 for the CU vein. See Appendix I for all forewing vein measurements as well as other Vogel's organs dimensions. The three characters relating to Vogel's organ are mapped onto a phylogeny of Satyrinae in Fig. 18. The adaptation of the phylogeny from Pena *et al.* (2006) was created using genera included in the comparative study and all other groups were removed for simplification. In most cases only one specimen per genus was examined.

The overall size of Vogel's organ (outer membrane surface area) ranged from 112.96 μm^2, in *Coenonympha*, to 2136.97 μm^2, in *Caligo*. Simple linear regressions using Vogel's organ dimensions and forewing length (see Appendix II) revealed a highly significant positive correlation between body size and the size of the Vogel's organ. Only the inner membrane of Vogel's organ length and width did not show a significant correlation with body size. The relationship between the outer membrane of Vogel's organ length and forewing length was highly significant ($R^2 = 0.8833$, $p < 0.001$, $n = 26$). A highly significant relationship was also found for the outer membrane of Vogel's organ

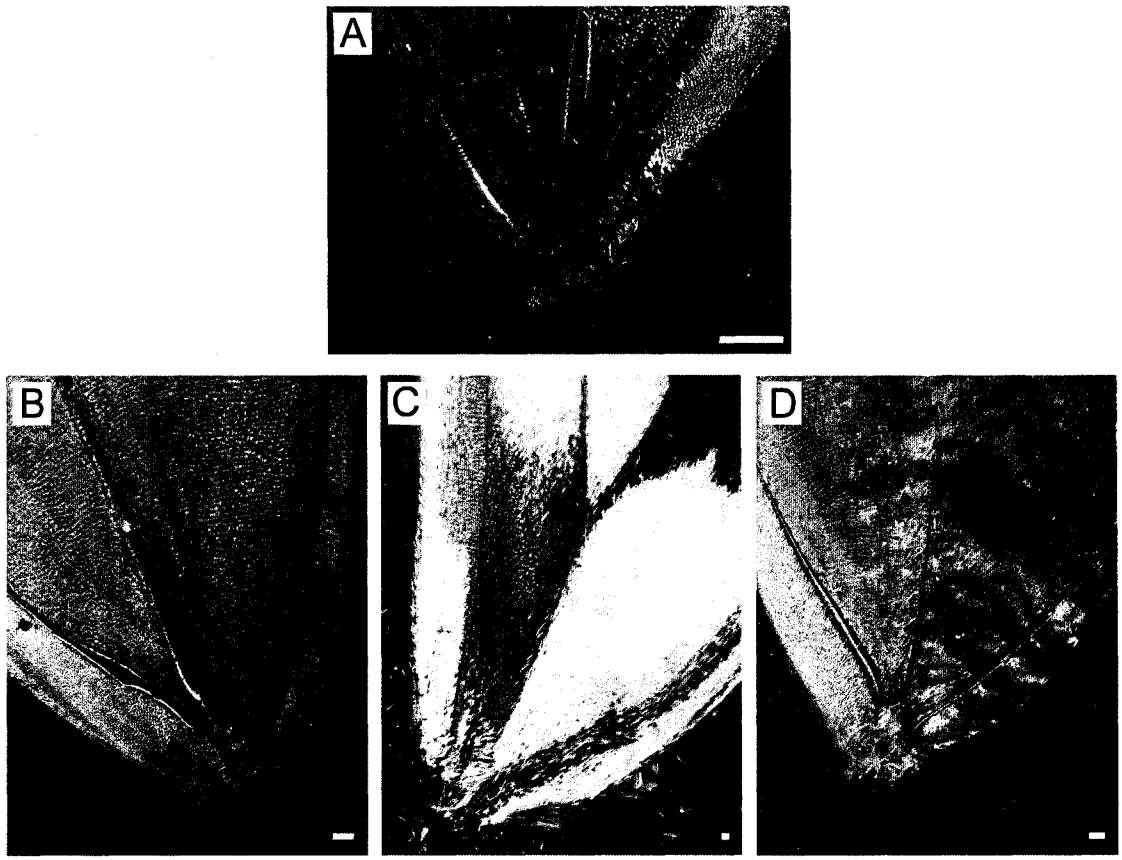

Fig. 17. Variation of forewing vein inflation. (A) High degree of inflation in all three of the forewing veins seen in *Maniola* sp. In contrast, three Satyrinae without substantial forewing vein inflation. (B) Left forewing of *Manataria* sp. (C) Right forewing of *Melanitis* sp. (D) Right forewing of *Gnophodes* sp. Scale bars = 500µm.

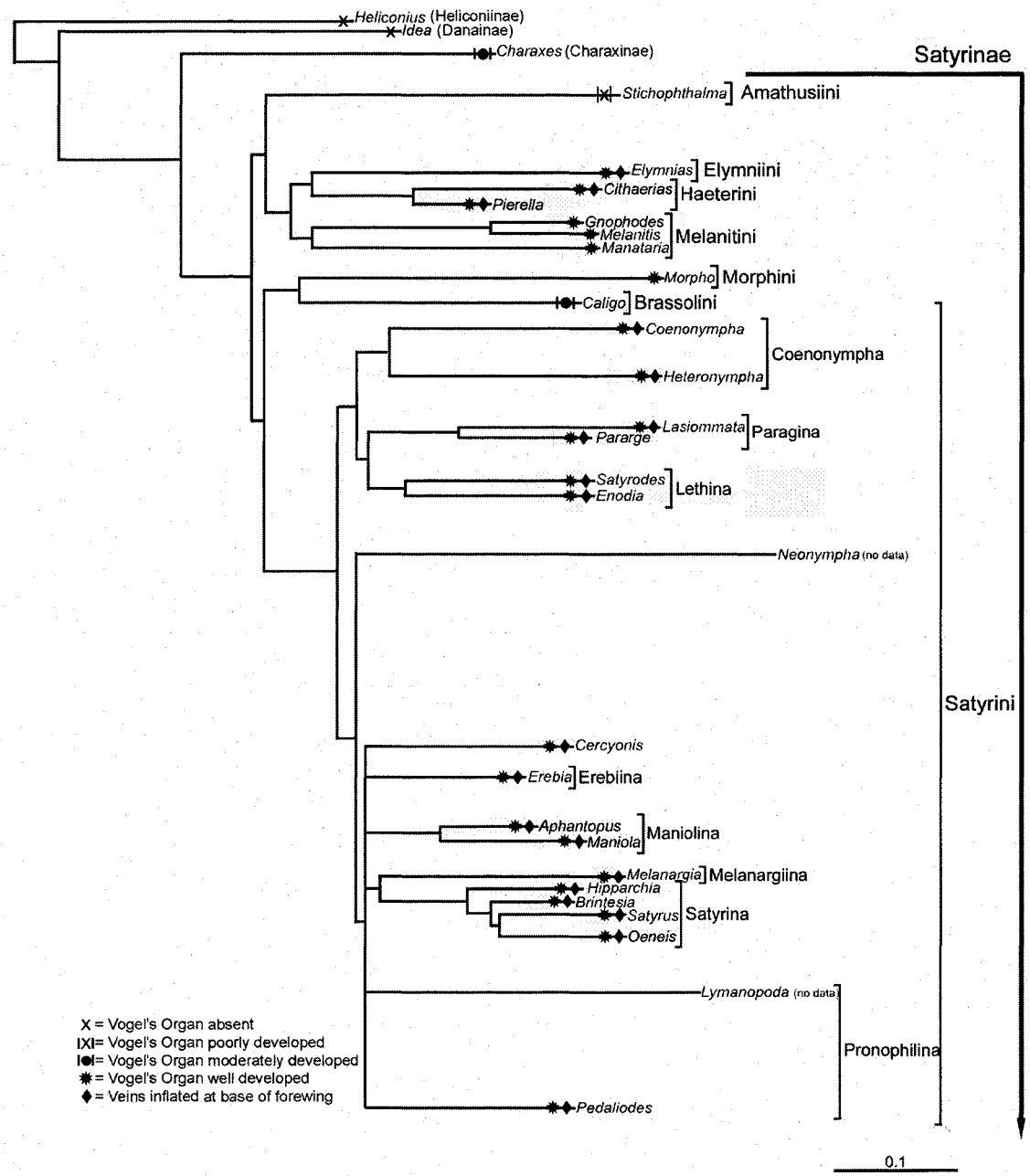

Fig. 18. Phylogenetic hypothesis for Satyrinae groups included in the comparative survey, adapted from Pena *et al.* (2006), with three Vogel's organ characters mapped on (see legend for symbol meanings).

width and forewing length ($R^2 = 0.9233$, $p < 0.001$, $n = 26$).

3.2 Physiology

3.2a *Pararge aegeria*

Audiograms were determined from extracellular, whole-nerve recordings from 9 female *P. aegeria* and 1 male *P. aegeria*. Neural activity was recorded from nerve branches NII & NIII together, or NIII alone. All animals were tested between 0.1 kHz and 25 kHz. Thresholds, based on the 'just noticeable difference' method, revealed an overall broadband pattern of peak sensitivities in the range of approximately 1 kHz – 18 kHz (Figs. 19&20), yielding a Q_{10} value of 0.192. The median audiogram showed a best frequency of 6.5 kHz with a median threshold of 70.4 ± 20.3 dB SPL and lowest threshold at 56.0 dB SPL. Low thresholds were also recorded for 3.5 kHz (median threshold of 73.1 dB SPL, lowest threshold of 67.9 dB SPL (n=8)) and 15 kHz (median threshold of 73.9 dB SPL, lowest threshold at 65.1 dB SPL (n=8)).

The latency of sensory and motor responses was determined for 1.5 kHz, 3 kHz, 6 kHz, and 13 kHz at intensities above threshold. Refer to Table 5 for the latency measurements. The sample size reflects the number of responses (per stimulus pulse) for one individual. The average sensory latencies ranged from 7.65 ± 0.80 ms (n =21) (1.5 kHz, +19 dB) to 17.07 ± 4.18 ms (n = 5) (3 kHz, +9 dB). The average latency of motor responses ranged from 39.71 ms ± 6.69 (n = 19) (3kHz, +23dB) to 74.38 ms ± 18.02 (n = 13) (6 kHz, +15 dB). A compound action potential resulting from 3 kHz sound stimuli

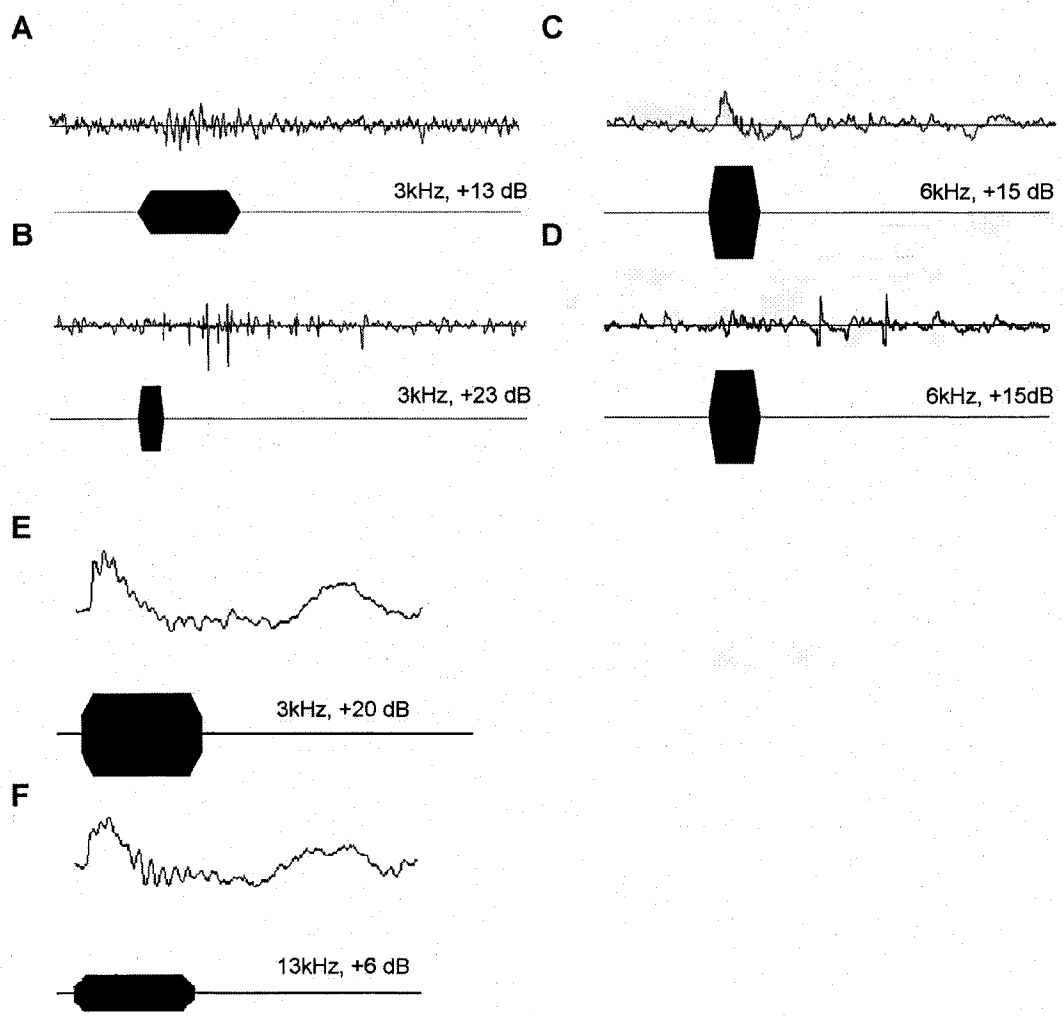

Fig. 19. Responses to low-frequency sound stimuli from whole-nerve extracellular recordings from NII and/or NIII in *Pararge aegeria*. All stimulus pulses are 30ms in duration and intensities are given relative to the threshold for given frequency.. (A) Sensory spikes and (B) motor spikes in response to 3kHz stimuli. (C) Sensory spikes and (D) motor spikes in response to 6 kHz stimuli. (E) Compound action potentials showing sensory and motor responses to 3 kHz stimuli and (F) 13kHz stimuli.

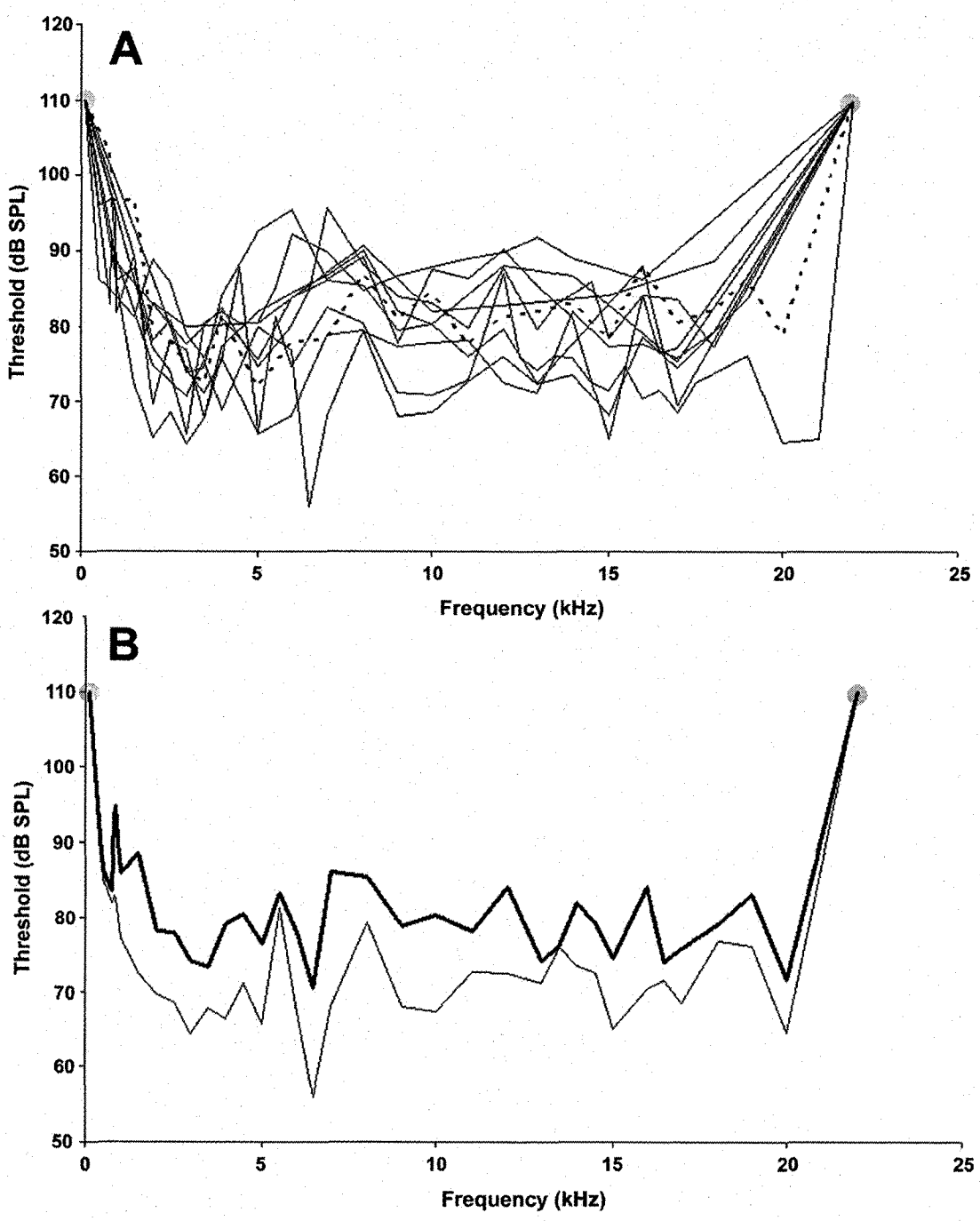

Fig. 20. Audiograms from 10 *Pararge aegeria* determined from extracellular whole-nerve recordings of IIN1c. (A) Individual thresholds (thin lines for 9 females, dashed line for 1 male). (B) Median thresholds represented by the bold line. The lowest thresholds are depicted by the thin line. Frequencies for which there was only n=1 for threshold determination were removed from the median and lowest threshold audiograms. Circled points at 0.1 kHz and 22 kHz are not actual thresholds, rather frequencies for which no threshold could be determined. Background noise of teh room during recordings was ~50 dB SPL.

Table 5. Mean response latencies of the IIN1c nerve of *P. aegeria* elicited by low-frequency sound stimuli.

Frequency	Intensity above threshold	Intensity in dB SPL	Latency (ms)	±	n=
Sensory response latency:					
1.5kHz	19	100	7.65	0.88	21
3kHz	+13dB	87	9.23	0.99	7
3kHz	+11dB	84	9.03	0.68	12
3kHz	+9dB	82	17.07	4.18	5
6kHz	+15dB	97	12.51	2.17	17
6kHz	+27dB	104	8.55	1.19	19
Motor response latency:					
1.5kHz	+19dB	100	52.08	11.23	24
3kHz	+23dB	102	39.71	6.69	19
6kHz	+15dB	97	74.38	18.02	13

reveals a sensory response with a latency of about 5.4 ms (Fig.18E). The compound action potential resulting from 13 kHz sound stimuli shows a sensory response latency of about 5.6 ms (Fig. 18F). For both compound action potentials, a broad peak is seen following the sensory peak, assumed to represent the motor response. The latency of the motor response, as measured from the compound action potentials, is approximately 52 ms for 3kHz and 13 kHz.

3.2b *Cercyonis pegala* and *Lasiommata megera*

Comparative physiology was carried out on the species most closely related to *P. aegeria* (according to Pena *et al.*, 2006), *Lasiommata megera* (Fig. 21A), which has a well-developed Vogel's organ (Fig. 21B). Preliminary evidence suggests a very responsive ear to low-frequency stimuli. Evidence of a typical sensory system intensity-response relationship can be seen in Fig. 21C, where increased stimulus intensity results in increased recruitment of sensory spikes. Audiograms were determined for 3 *L. megera* (1 male, 2 sex unknown) (Fig.21D). The ears were broadly tuned to frequencies in the range of 0.5 kHz to 18 kHz. The median audiogram reveals best frequencies at both 4 kHz and 10kHz, with median thresholds of 71 dB SPL, testament to the broadband nature of the tuning of this ear.

Recordings of *Cercyonis pegala* (sex unknown) yielded only one audiogram (Fig. 22). Preliminary results show that the ear of *C. pegala* appears to be broadly tuned to low-frequency sounds in the range of 0.5 kHz to 15 kHz.

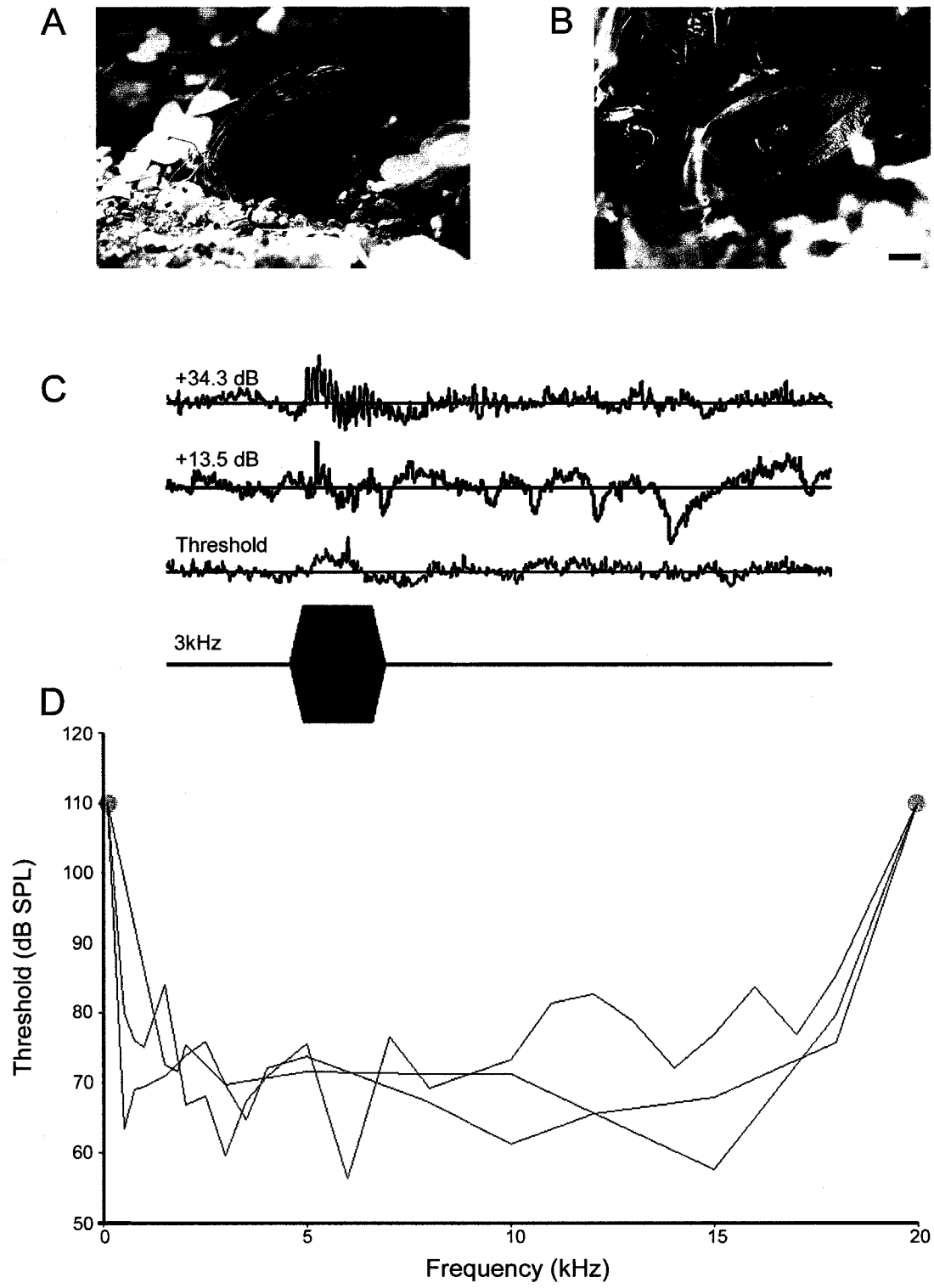

Fig. 21. Comparative physiology of a close relative of *P. aegeria*. (A) Live *Lasiommata megera* (Satyrinae). (B) Vogel's organ of *L. megera*. Scale bar, ~100μm. (C) Intensity-response relationship, showing increased sensory response to 3 kHz sound stimulus with increased intensity, determined from extracellular recordings of IIN1c. (D) Audiograms for 3 *L. megera*. Circled points at 0.1 kHz and 20 kHz are not actual thresholds, rather frequencies for which no threshold could be determined.

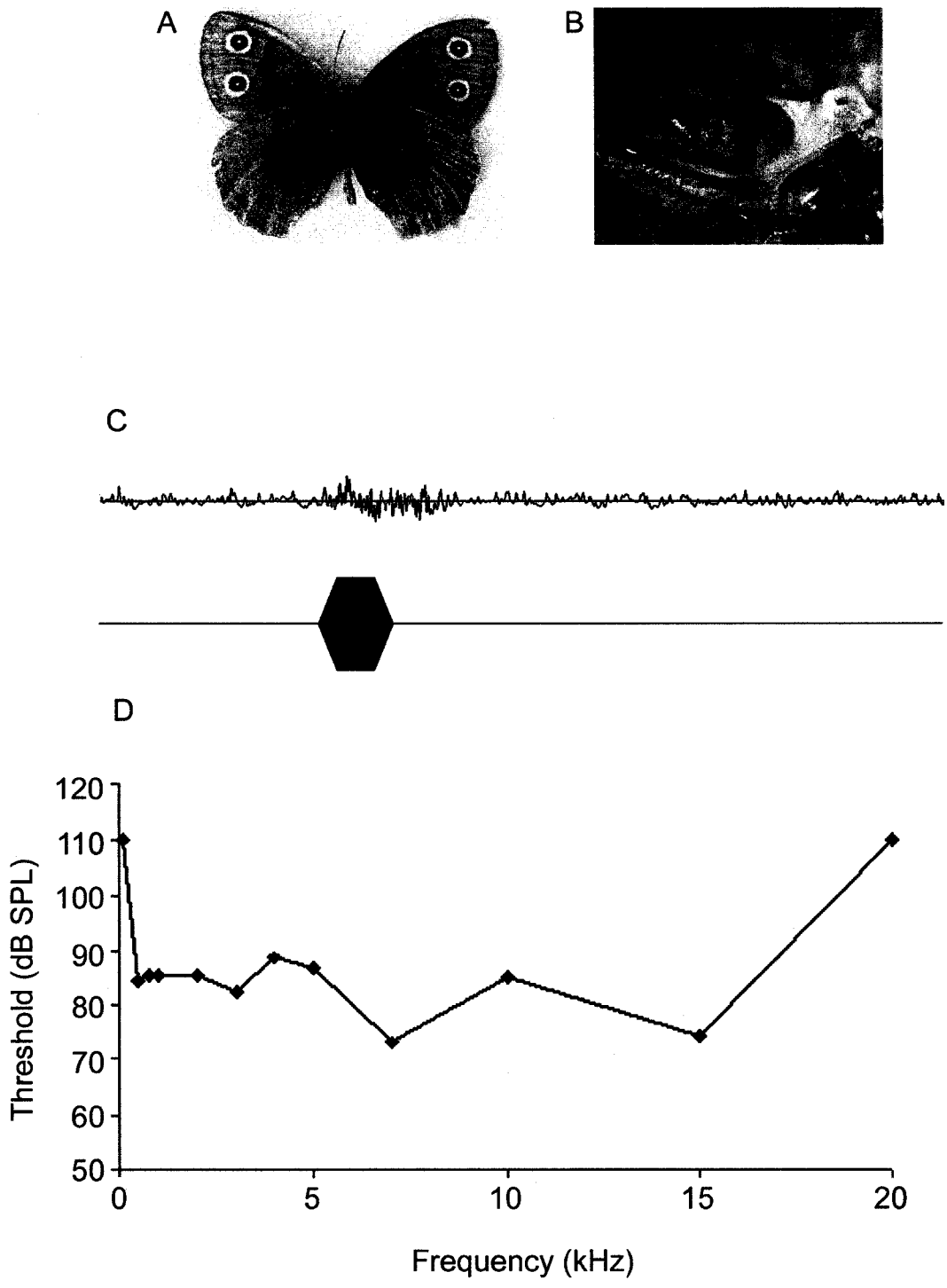

Fig. 22. Comparative physiology of *Cercyonis pegala*. (A) Ventral view of a dried *Cercyonis pegala* specimen. Photo credit, N. Wahlberg. (B) Close-up view of the well developed Vogel's organ of *C. pegala*. Scale bar, ~100μm. (C) Extracellular whole-nerve recordings of the IIN1c yielded sensory spikes in response to 1 kHz stimuli, at an intensity above threshold. (D) Audiogram for one *C. pegala* (sex unknown). Points at 0.1 kHz and 20 kHz are not actual thresholds, rather frequencies for which no threshold could be determined.

3.3 Behaviour

3.3a *Behavioural Response to Sound*

3.3a.i Tethered – Ten *P. aegeria* were stimulated with sound stimuli in the range of 0.5 kHz to 30 kHz. Of the 10 butterflies, possible behavioural responses were detected in 8 (5 male, 3 female) individuals. In preliminary trials, using intense ultrasound, no response was observed.

Responses to sound stimuli included the closing of wings, pulling wings back (twitching), flapping of wings, proboscis lifting, abdomen lifting, antennae twitching, and cessation of movement. Responses were elicited over a broad range of frequencies (0.75 kHz to 18 kHz). The best response was found at a frequency of 3 kHz at 88 dB SPL. In cases where a response was observed, no response could be elicited following ablation of the Vogel's organ. However, caution must be exercised in the interpretation of these results, as responses were not always consistent and very intense sound (>80 dB SPL) was required.

3.3a.ii In-flight - Free-flying *P. aegeria* (n =5) subjected to ultrasonic pulses produced by the electronic dog whistle did not exhibit any detectable behavioural response. Once stimulated with ultrasound, the butterflies continued to fly in an uninterrupted manner. Preliminary testing of an in-flight response to low-frequency sounds did not yield a behavioural response. *P. aegeria* (n = 3), within the flight cage, did not appear to respond to low-frequency sound stimuli while flying within the cage. However, it was difficult to induce constant flight within the cage.

3.3b *Sound Production*

While recording *P. aegeria* during natural flight, no ultrasound could be detected using the bat detector. Furthermore, during observations of *P. aegeria* freely flying no audible sound production was ever detected.

3.4 Bird flight recordings

Preliminary recordings of chickadees flying up to a handful of seed revealed that most of the energy of the flight sounds fell under 15 kHz, at a distance of about 5-10cm (Fig. 23). Recordings of 8 chickadees flying up to a feeder showed that the flight sounds are broadband, ranging from <100 Hz to just below 22 kHz for a bird flying in towards the feeder from a distance of 30cm. The intensities of these sounds ranged from 64.08 to 80.31 dB SPL for 3 kHz. For 5 kHz, the intensities ranged from 60.33 to 77.30 dB SPL. For 10 kHz, the intensities ranged from 61.05 to 80.56 dB SPL.

Recordings of 15 grackles flying up to a feeder showed that these flight sounds are also broadband in nature – with frequencies ranging from <100 Hz to just below 22 kHz (Fig. 24). The intensities of these sounds ranged from 73.07 to 86.88 dB SPL for 3kHz. For 5 kHz, the intensities ranged from 69.13 to 83.34 dB SPL. For 10 kHz, the intensities ranged from 69.67 to 87.19 dB SPL.

See Table 6 for a complete list of the intensities for varying frequencies and distances for both the chickadee and grackle recordings.

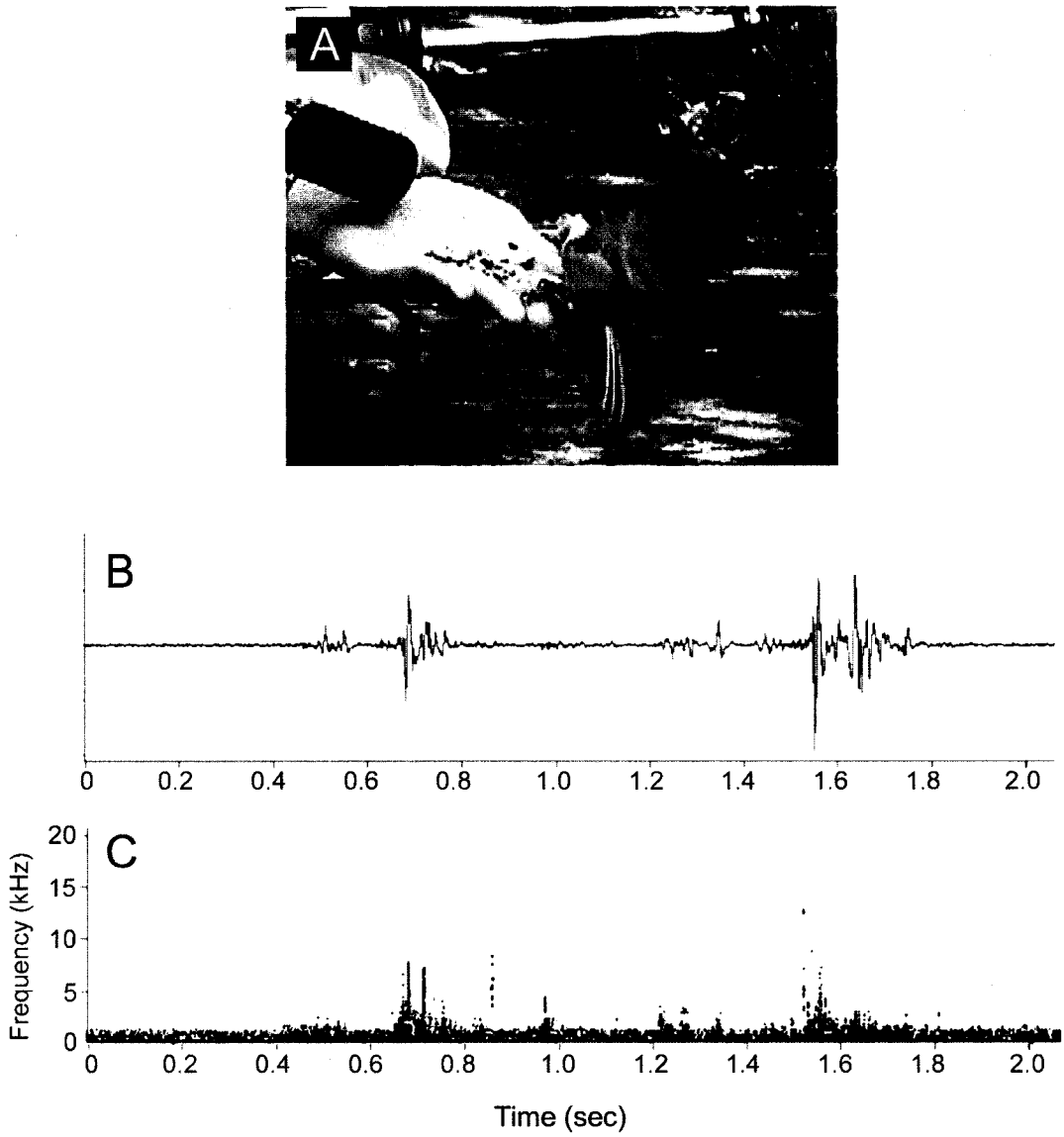

Fig. 23. Preliminary bird flight recordings achieved by capturing the sound of chickadees flying up to a microphone held in a hand full of seed. (A) Video frame of chickadee flying in towards microphone and landing on hand. (B) Waveform for representative flight recording of an individual chickadee flying in towards the microphone and then away from the microphone. (C) Spectrogram for the above waveform, showing energy of the sounds falling under 15 kHz.

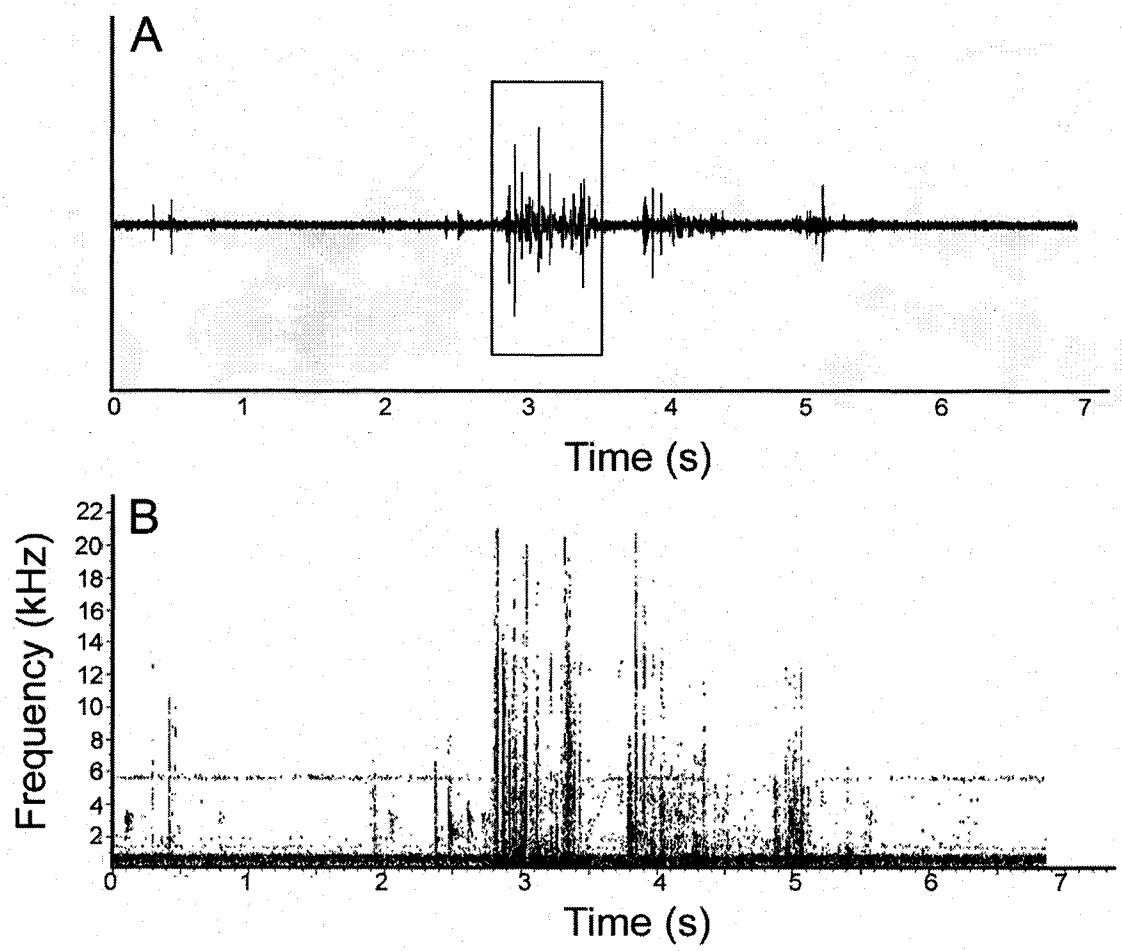

Fig. 24. Bird flight recordings achieved by capturing the sound of grackles (*Quiscalus quiscula*) flying up to a microphone. (A) Waveform for a representative flight recording of an individual grackle flying in towards the microphone from a distance of approximately 50 cm. The square outlines the portion of oscillogram where the bird is flying towards microphone. (B) The accompanying spectrogram indicates energy of the flight sounds falling under 22 kHz.

Table 6. Maximum intensities (given in dB SPL) of grackle and chickadee flight sounds calculated at distances of 2 cm and 14 cm.

	1 kHz		3 kHz		5 kHz		10 kHz		15 kHz		18 kHz	
	2 cm	**14 cm**	**2 cm**	**14 cm**	**2 cm**	**14 cm**	**2 cm**	**14 cm**	**2 cm**	**14 cm**	**2 cm**	**14 cm**
Grackle	92.76	92.06	86.26	86.88	83.34	82.66	82.94	87.19	74.57	80.44	61.77	66.27
Chickadee	85.72	84.57	80.01	80.31	77.30	76.22	76.62	80.56	68.45	65.86	56.14	56.87

4. Discussion

Despite an abundance of indirect and anecdotal evidence suggesting that hearing is widespread in Nymphalidae, specifically Satyrinae, experimentally supported evidence is surprisingly lacking. Using a representative satyrid, *Pararge aegeria*, this study presents the first comprehensive investigation of hearing in a Satyrinae butterfly, by exploring the three lines of evidence for hearing: (1) morphological, (2) physiological, and (3) behavioural. The Vogel's organ in *P. aegeria* appears to fulfill most criteria of a typical insect ear. In this discussion, several hypotheses for the function of Vogel's organ in *P. aegeria* are considered. The results of this study support the hypothesis that Vogel's organ in *P. aegeria* functions in the detection of avian predators. An examination of Vogel's organ throughout Satyrinae reveals that well-developed Vogel's organs are concentrated within Satyrinae. Implications of these findings for the evolution of hearing in butterflies are addressed.

4.1 Is the Vogel's organ in *Pararge aegeria* an ear?

Vogel's organ is widespread throughout Nymphalidae and variable in form (refer to Table 1). An auditive function for Vogel's organ has been suggested based on early anatomical work (Vogel, 1912; LeCerf, 1926; Bourgogne, 1951), but this organ has rarely been tested directly for its involvement in hearing. Just three studies have provided experimental evidence for hearing, using *Erebia* sp. (Satyrinae) (Ribaric & Gogala, 1996), *Manataria maculata* (Satyrinae) (Rydell *et al.*, 2003) and *Hamadryas feronia* (Biblidinae) (Yack *et al.*, 2000).

Evidence for hearing in insects may be obvious in cases where the animal is communicating with sonic sounds and features a conspicuous hearing organ. For

example, crickets communicate with low-frequency sounds audible to humans and receive signals *via* prominent tympanal ears (reviewed in Yager, 1999). However, there are cases where evidence for hearing may not be as apparent. Several insects lack noticeable external manifestations of ears. Drepanid moths have discreet tympanal ears on their abdomens (Surlykke *et al.*, 2003). Similarly, mantids have concealed hearing organs on the metathorax (Yager, 1990). On the other hand, there are also cases of insects demonstrating a physiological response to sound, yet no evidence of a hearing organ. In these cases, thresholds are often very high (+90 dB) and likely a by-product of stimulating a non-auditory chordotonal organ that actually functions in proprioception (Yack & Fullard, 1993a & b). Therefore, defining what constitutes an insect ear requires clarification.

Yack & Fullard (1993a) advise that to conclude that a sense of hearing exists, three important criteria must be met. These criteria include (1) the presence of a morphologically differentiated receptor system, including a chordotonal organ which is characteristic of all insect auditory systems to date (Yack, 2004), (2) physiological evidence that the receptor is sensitive to biologically relevant sound frequencies and intensities, and (3) the reputed ear must facilitate an adaptive behavioural response to sound. This study attempts to answer the question of whether or not the Vogel's organ in *P. aegeria* meets the conditions of a functional ear.

Morphologically, the Vogel's organ of *P. aegeria* resembles a typical tympanal ear. In accordance with most tympanal ears, the Vogel's organ is a paired structure, consisting of an oval thinned membrane backed by a large tracheal air sac, and is associated with what appears to be sensory structures. The thickness of the thinned

region of cuticle remains to be determined. However, it is translucent to opaque, which is characterstic of most low-frequency ears, such as those seen in *H. feronia* (Yack *et al.*, 2000). Most ultrasound-sensitive ears have thinner transparent membranes, as seen in the hedylid, *Macrosoma heliconaria* (Yack & Fullard, 2000) and other moths (Minet & Surlykke, 2003).

The membrane of the Vogel's organ in *P. aegeria* is not homogenous, but rather is composed of two distinct regions. SEMs reveal that the two regions, the outer and inner membranes, differ in surface topography. The outer membrane is covered with ripples, while the smooth inner membrane is covered with small setae. Dissimilar membrane surfaces could be indicative of differential vibrational properties. It appears as though the inner membrane is innervated by one chordotonal organ while two other chordotonal organs innervate the outer membrane. These structures are assumed to be chordotonal organs based on corresponding innervation patterns and location to the chordotonal organs reported in *H. feronia* (Yack *et al.*, 2000). This design could reflect capabilities for frequency discrimination based on the "place principle" of frequency analysis as described in Acrididae (grasshoppers) (reviewed in Yack, 2004). The difference surface topographies of the inner and outer membranes may impart different vibrational qualities to the membranes, although this remains to be determined.

Extracellular recordings reveal auditory responses from only the nerve branches directly innervating the ear (NII, NIII), supporting Vogel's organ as the site of sound reception. Responses could only be obtained by recording from NII and NIII together, or NIII alone. Despite the fact that NII has a direct connection to the tympanal membrane, the physiology data provides evidence that the putative chordotonal organs associated

with NIII play a critical role in sound reception. Auditory responses were obtained from physiological recordings of NII in *Hamadryas feronia*, which contributed to confirming the role of Vogel's organ in hearing (Yack *et al.*, 2000).

The latencies of the physiological responses recorded for *P. aegeria* are comparable to other auditory systems, supporting Vogel's organ function in hearing. Latencies measured were as low as 5.4 ms and are comparable to other sensory latencies in insects ears of 5-6 ms at higher intensities (Göpfert & Wasserthal, 1999). Whole nerve recordings from cricket tympanal nerves yield latencies of 6-7 ms at intensities of 70-80 dB SPL (Imaizumi & Pollack, 2001). The latencies measured in *P. aegeria* are of a conservative nature as the first sensory spikes were difficult to identify amongst the high background noise. It is more likely that the time from the onset of the stimulus to the point where several auditory spikes had been recruited was actually measured. Based on this, latencies measured from the compound action potentials may be considered more accurate as the onset of the sensory response is more prominent.

The physiology data supports *P. aegeria* demonstrating neural sensitivity to sounds of a biologically relevant nature. The lowest threshold determined for *P. aegeria* was 56.0 dB SPL at 6.5 kHz. At first glance, the thresholds of *P. aegeria* appear to be slightly high in comparison to other insect ears. The majority of tympanal insects have best sensitivity in the range of 30-50 dB SPL (Yager, 1999). However, there are many cases of insect ears with "higher thresholds" including mantids (60-70 db SPL) (Yager, 1999), scarab beetles (55-70 dB SPL) (Forrest *et al.*, 1997), and lacewings (55-60 dB SPL) (Miller, 1984). These auditory systems, including *P. aegeria*, with higher

thresholds are still fully functional as they are capable of receiving sounds of biologically relevant intensities.

The high thresholds of *P. aegeria* may also be explained by technical problems. The nature of this prep caused great difficulty in obtaining successful recordings. The small size of *P. aegeria* meant that there was little room for the electrode to be manipulated within the recording site. As a result, contact between the recording electrode and very fine nerve branch may have been compromised. Substantial background noise was produced during recordings, such that the signal was often not even detectable. Recordings with double-hook electrodes were attempted in an effort to increase the signal:noise ratio. The level of background noise did not significantly diminish, however, the double-hook electrode was too large for the recording site which made it difficult to establish adequate contact with the nerve branch. It is suggested therefore, that the thresholds presented in this study represent a conservative estimate. Nevertheless, despite the seemingly high thresholds determined for *P. aegeria*, the thresholds still do match the bird flight sound intensities (see following section).

The last aspect of testing a sense of hearing is behavioural. Trials using tethered *P. aegeria* revealed marginal responses to low-frequency sounds. The butterflies responded to loud sounds with various wing movements, proboscis lifting, abdomen lifting, antennae twitching, and cessation of movement. Similar responses have been observed in other hearing insects (e.g. Ribaric & Gogala, 1996). However, in the case of *Pararge*, the function of these responses is questionable. The best behavioural threshold recorded was 88 dB SPL for 3 kHz. Given the high intensities required to elicit behavioural responses, it could be that they are actually the by-product of proprioceptor

stimulation. At this point, behavioural evidence for hearing in *P. aegeria* is lacking. Behavioural responses to sound are notoriously difficult to demonstrate for a number of reasons. Consideration into the function of this ear may provide insight into methodology modifications that may facilitate more consistent and convincing behavioural responses. Refer to the following section and possible explanation for discussion of the lack of definitive behavioural response in *P. aegeria*.

4.2 What is the function of hearing in *Pararge aegeria*?

Morphological and physiological evidence has been presented to support Vogel's organ in *Pararge aegeria* as a functional hearing organ. Assuming Vogel's organ is an ear, possible functions must be addressed. First, it is possible that it functions in conspecific communication. An essential element of an acoustic system involving conspecific communication is the presence of sound production. Sound production has been reported in several nymphalid butterflies (see Table 1), including 2 Satyrinae species, *Pharneuptychia* nr. *pharnaba* (Kane, 1982) and *Yphthimoides castrensis* (Murillo-Hiller, 2006). However, there is no evidence to date that *P. aegeria* produce sounds. Behavioural trials revealed no evidence of low-frequency or ultrasonic sound production. In addition, of all the researchers who have worked with this species, there has never been a report of sound production, even under natural field conditions. Based on absence of sound production it is proposed that the Vogel's organ of *P. aegeria* does not function in conspecific communication.

Second, it is possible that the Vogel's organ of *P. aegeria* functions in bat detection. In addition to a great number of moths have been shown to possess bat-detecting ears, the nocturnal hedylid, *Macrosoma heliconaria*, has an ultrasonic, bat-

detecting ear. Hedylids perform classic evasive flight maneuvers in response to ultrasound, an adaptive response to predation by bats (Yack & Fullard, 2000; Yack *et al.*, submitted). Likewise, the crepuscular Satyrinae, *Manataria maculata*, exhibits evasive flight in response to ultrasound (Rydell *et al.*, 2003). If a hearing system evolves to function in bat detection, significant selection pressure from bats must exist. There are no reports of *P. aegeria* being active at times when predation by bats is likely. Furthermore, physiology and behavioural data demonstrate no sensitivity to ultrasound. Based on this evidence, the prospect that this Vogel's organ is a bat detector is discounted.

Third, it is hypothesized that the Vogel's organ of *P. aegeria* is designed to receive the sounds of bird flight. This hypothesis has never been proposed with regards to butterfly hearing. Predictions to support this hypothesis are (1) birds must prey on *P. aegeria*, (2) birds must produce sound when attacking butterflies, (3) these sounds produced by birds must match the sensitivity of hearing in *P. aegeria*, and (4) *P. aegeria* must exhibit an adaptive behavioural response to these sounds. These predictions are discussed below.

First, birds do prey on *P. aegeria*. *P. aegeria* are diurnal, exposing them to the risk of bird predation. Observations of birds preying on butterflies in general have been reported for butterflies both on the wing and at rest (Dover, 1920; Wheeler, 1934; Carpenter, 1933, 1941; Collenette, 1935; Shapiro, 1977; Muyshondt & Muyshondt, 1976; Wourms & Wasserman, 1985; Chai & Srygley, 1990; Sargent, 1995). It has been communicated that cases of birds attacking, or attempting to attack *P. aegeria* do occur and beak marks on the wings of this species have been observed (Martin Jones,

Manchester, UK, pers. comm.). Also, *P. aegeria* are considered a palatable species based on a larval diet of grasses. Thus, a mechanism for keen predator detection such as hearing would be beneficial (Scott, 1986; Gibbs *et al.*, 2004).

Next, it is predicted that birds produce sounds during flight while attacking a butterfly. Recordings of chickadees and grackles, known insectivores, revealed that the birds produce very audible flight sounds. The intensities of these sounds are as high as 92.76 dB SPL at 1 kHz at a distance of 14cm to 2 cm. Analysis of the flight sounds revealed significant energy in the range of 0.1 kHz to 15 kHz.

It is also predicted that the flight sounds of birds must match the hearing sensitivity of *P. aegeria*. Audiograms show that *P. aegeria* are sensitive over a range of 1 kHz to 18 kHz, which matches energy of the bird flight sounds. Additionally, the high thresholds of *P. aegeria* closely match the high intensities of the flight sounds, which had a maximum intensity of 92.76 db SPL at 1 kHz at a distance of 14 cm to 2 cm. Conceivably, the high hearing threshold of *P. aegeria* may allow for the distinction between the loud sounds of avian predators and the ambient background noise of the environment. Lastly, the ears of *P. aegeria* are broadly tuned, which is characteristic of hearing systems designed for predator detection. Low Q_{10} values (as seen in *P. aegeria*) are correlated with broadly tuned auditory systems, such as reported for the auditory T-cell of katydids that functions in bat detection (Q_{10} value <1.0) (Faure & Hoy, 2000). Similarly, hawkmoths (Q_{10} values from 0.41 - 0.64) also have auditory systems functioning in bat detection (Göpfert & Wasserthal, 1999). In contrast, finely tuned ears have higher Q_{10} values, for example in cicadas (Q_{10} value of 2.1) which use hearing for communication (Munch, 1999 cit. in Hennig *et al.*, 2004). The broadly tuned nature of

the *P. aegeria* Vogel's organ contrasts the finely tuned ears of *H. feronia* that is suggested to function in conspecific communication (Yack *et al.*, 2000). This disparity in tuning between the two Vogel's organ likely correlates with a diversity in function, supporting the function of the Vogel's in *P. aegeria* as a bird detector.

If *P. aegeria* are listening to birds then it is predicted that they would exhibit an adaptive behavioural response to sounds with spectral similarity to bird flight sounds. It was anticipated that *P. aegeria* would respond to sound by flying away. The behavioural component of this study using tethered individuals did not yield results expected to fulfill this prediction. *P. aegeria* demonstrated inconsistent behavioural responses to only high intensity sounds. Similarly, *Cercyonis pegala* respond to only high intensity sounds (> 95 dB SPL) in the form of antennal flicking and other wing movements including flying (Frings & Frings, 1956). Preliminary physiology study of *C. pegala* revealed that both of these Satyrinae possess similar hearing sensitivity, suggesting they both function in a similar manner. It is possible that both Satyrinae are adapted for receiving high intensity sounds stimuli from their environment. The physiology data provides some evidence of a behavioural response to sound in the form of motor spikes, which could be indicative of an escape response. The latency of these motor spikes ranged from 39.71 ms to 74.38 ms, which is comparable to latencies of startle responses reported in the moth, *Achroia grisella* (~50 ms) (Greenfield & Baker, 2003).

It is possible that in order to elicit a behavioural response, several stimuli may be required in conjunction or stimuli may require a specific context. The response of *P. aegeria* to low-frequency sounds may be multi-modal. Different forms of stimuli produced in succession may be required to trigger a behavioural response. Certain moths

are known to use both ultrasonic and chemical stimuli during courtship (Conner, 1999). Examples of multi-modal systems are also found in cockroaches (Ritzmann *et al.*, 1991), the predators of many aposematic insects (Rowe & Guilford, 1999 & ref. within), and many higher vertebrates (Partan & Marler, 1999). Some of these multimodal systems necessitate two or more specific stimuli presented in conjunction to elicit a response (Partan & Marler, 1999). This could sufficiently explain why sound stimuli alone induced inconsistent responses and required high intensity sound levels in *P. aegeria.*

Consideration to the context within which the sound stimuli occurs must also be given. In the case of *Erebia*, the best behavioural responses occur when the butterflies roost in groups (Ribaric & Gogala, 1996). Similarly, the crepuscular *Manataria maculata* only responds to ultrasound at dusk and dawn (Rydell *et al.*, 2003). It may be adaptive for *P. aegeria* to respond to very specific stimuli under restricted contexts. *P. aegeria* have broadband auditory sensitivity and may require additional cues to induce a response in order to extract relevant sound stimuli from all other background noise of the environment. *P. aegeria* may respond to specific, complex sounds rather than the pure-tone sounds used in this study. The experimental conditions, such as nature of the tether, may have also hindered a behavioural response. It would be most beneficial to test for a behavioural response under natural conditions, such as in the field or use more specific sound stimuli, such as playbacks of the flight sounds.

Another possible function of hearing in *P. aegeria* is to detect the calling songs of birds and respond by remaining stationary and camouflaged in the presence of danger. Although this study did not address bird calls, this could be explored in further studies by assessing flight tendency in the presence and absence of bird calls. It may be difficult to

detect a response if the response is cessation of movement. Inhibition responses are not uncommon in acoustic insects and these responses take on a variety of forms. Inhibition responses may include call inhibition as seen in the acoustically communicating moth, *Achroia grisella*, in response to gleaning bat echolocation calls (Greenfield & Baker, 2003). As suggested for *P. aegeria*, inhibition responses may simply comprise cessation of movement, as seen in stick insects which respond to threats by freezing their body position (Burrows & Morin, 2002). Inhibition responses function in reducing conspicuousness, be it in the form of staying quiet or hidden in the presence of a predator, which may be the best strategy for *P. aegeria*

Based on the fulfillment of 3 out of the 4 predictions outlined here, there is support for the hypothesis that *P. aegeria* are using a sense of hearing for the detection of avian predators.

4.3 Consideration of the diversity of hearing in Satyrinae and the evolution of hearing in butterflies

The comparative portion of this study was carried out to determine how widespread the Vogel's organ is within Satyrinae, using the recent phylogeny provided by Pena *et al.* (2006). A focus on variation in structure and size of Vogel's organ was incorporated in the expectation that this may provide insight into functional diversity. Comparative physiology was carried out on two other Satyrinae to determine if they match the sensitivity of *P. aegeria*. The results of the comparative study of hearing in Satyrinae may provide insight in the evolution of hearing in butterflies, and results will be discussed under this conjecture.

The Vogel's organ appears to be distributed through Satyrinae (Otero, 1990a), but a systematic examination of its presence and/or degree of variation was not reported until now. For the comparative study, representatives were taken from each clade of Satyrinae and outgroups from other Nymphalidae subfamilies (according to Pena *et al.*, 2006). It was found that, without exception, all members of the subfamily Satyrinae featured a Vogel's organ. Most of the Satyrinae had well developed Vogel's organs with an inner membrane and inflated veins. However, there were some exceptions to this trend. Three of these exceptions are species that have just recently been placed in Satyrinae, originally classified within their own subfamily Morphinae. This suggests that this current placement may require further modification or selection pressures have caused modification of Vogel's organ. *Caligo* (Brassolini) and *Stichophthalma* (Amathusiini) were the only two Satyrinae included in this study that did not have a well developed Vogel's organ and vein inflation. Another anomaly was seen in *Morpho* sp. (Morphini), which was found to possess a well-developed Vogel's organ, but no inflated forewing veins.

Three members of the tribe Melanitini (Satyrinae) also presented an interesting case with a lack of forewing inflation. The tropical *Gnophodes*, *Melanitis*, and *Manataria* are all crepuscular and are often found roosting in congregations in shaded areas (Pena, 2004). As previously discussed, *Manataria maculata* respond to ultrasound, presumably a defence against insectivorous bats. Given the close relation and similar life history, it is proposed that all three species have ultrasound sensitive Vogel's organs. The typical inflated veins of Satyrinae may contribute to auditive function through association with the tracheal system. Air pressure in the tracheal system affects the

displacement of the tympanal membrane, influencing the auditory properties of the hearing organ (Meyer & Hedwig, 1995). The degree of inflation of the forewing veins in Satyrinae could impact the air pressure of the tracheal system and thus the tuning of the Vogel's organ. In bushcrickets, modification of the tracheal system by closing spiracles increases sensitivity to lower frequency sounds (Romer & Bailey, 1998). Inflated veins may modify the tracheal system in a manner that increases sensitivity to lower frequency sounds. This could lend explanation to the non-inflated veins of the proposed three ultrasound-detecting Satyrinae.

From a quantitative perspective, the diversity in the dimensions of Vogel's organ was high and this was found to be positively correlated with body size. A similar trend is seen for the tympanal ears of noctuid moths (Surlykke *et al.*, 1999). This diversity in size of Vogel's organ could reflect a diversity of sensitivity and tuning. Future physiological study of Vogel's organ throughout Satyrinae may confirm this.

Comparative physiology of *Lasiommata megera* revealed similar trends in sensitivity to *P. aegeria*. Both species exhibit broadband tuning in the same frequency range. This similarity was expected as their Vogel's organs are nearly identical (morphologically) and they share similar habitats. Recordings from *Cercyonis pegala* also revealed a similar, broadly tuned auditory system. Similar auditory sensitivity shows that hearing in *P. aegeria* is not an isolated phenomenon and suggests that these three Vogel's organ may function in a similar manner.

Looking at Satyrinae as a whole, one must question why this group warrants consistent well-developed Vogel's organs. Satyrinae are typically found at the understorey level, where light levels are low (Fermon, 2002). Residing in this habitat, the

visual acuity of Satyrinae may be obstructed by foliage and branches and reduced by low light levels. Decreased visibility may have increased selection pressure for the development of alternate senses, such as hearing, for the detection of predators. In the palatable Satyrinae, vulnerability to predators is presumably high as they possess no known chemical defences and have a larval diet of grasses. Also, the colouration of most Satyinae suggests a design for camouflage. It is possible that Satyrinae rely on the acoustic cues of predators because sounds are more easily detected from inconspicuous positions than visual cues.

Although the function of Vogel's organ in Satyrinae has yet to be confirmed, there does appear to be a strong selection pressure for hearing in this group. Further investigation of hearing in Satyrinae should focus on the clades not represented in this study. For example, no data has been provided here for the subtribe of Satyrini, Euptychiina. However, the only two cases of sound production in Satyrinae, *Pharneuptychia* (Kane, 1982) and *Ypthimoides* (Murillo-Hiller, 2006) are both found in this subtribe. Euptychiinae is an excellent candidate for further comparative study of Satyrinae hearing. Overall, the subfamily Satyrinae provides an interesting opportunity for the investigation of Vogel's organ and its potential role in hearing in a geographically, anatomically, and behaviourally diverse group.

Looking at Vogel's organ from a broader perspective, this character appears to only occur within Nymphalidae, yet varies in presence and degree of development. The basal subfamily, Danainae, includes *Idea* sp. which completely lacks a Vogel's organ. LeCerf (1925) cites Danainae as a group lacking a tympanal organ. This suggests that absence of Vogel's organ is the primitive condition. However, *Heliconius* sp.

(Heliconiinae) also displays complete absence of Vogel's organ and it belongs to one of the more derived Nymphalidae subfamilies (Wahlberg *et al.*, 2003). Three other nymphalid genera were found to have Vogel's organ in poorly or moderately developed states. *Charaxes* sp. (Charaxinae), the most basal of the three, has a moderately developed Vogel's organ. *Stichophthalma* sp. (Amathusiini, Satyrinae) has a poorly developed Vogel's organ and is rather primitive compared to the rest of Satyrinae. *Caligo* sp. (Brassolini, Satyrinae) features a moderately developed Vogel's organ. Interestingly, *Caligo* forms a sister group with *Morpho* (Morphini, Satyrinae), which features a highly developed Vogel's organ. It is difficult to speculate why *Caligo* is relatively under-developed without more information regarding life history traits and other genera belonging to these groups. *Caligo* is crepuscular (Pena, 2004), and perhaps the moderately developed Vogel's organ is some form of ultrasonic bat detector.

The Vogel's organ is highly variable and this study strongly supports Satyrinae as the only group that consistently has a well-developed Vogel's organ. The family Nymphalidae displays a large degree of variation in Vogel's organ with no clear phylogenetic pattern and a variety of selections pressures must be at work. To date, Vogel's organ has been reported only within Nymphalidae, varying widely in presence and morphology. This variation could be a reflection of a diversity of functions. Further comprehensive studies of butterfly hearing are required to confirm the possible many functions of Vogel's organ.

The Vogel's organs of Nymphalidae appear to be homologous based on innervation patterns of the forewing. Akin to other forewing bases in Lepidoptera, the Vogel's organ of *P. aegeria* is innervated by a branch of nerve IIN1c (Minet & Surlykke,

2003). Vogel (1912) provided one of the only detailed accounts of the innervation of Vogel's organ, using *Ephinephele jurtina* (Satyrinae). The innervation of *Hamadryas feronia* is also described in-depth by Yack *et al.* (2000). *P. aegeria*, *E. jurtina*, and *H. feronia* all possess three branches (NI, NII, NIII) of mesothoracic branch IIN1c which project up the forewing veins. These branches follow similar patterns in all three butterflies, where NII and NIII innervate chordotonal organs that appear to be associated with Vogel's organ.

Looking at hearing in butterflies as a whole, the tympanal ear found in the hedylid butterflies must be considered. Hedyloidea are the proposed ancestral group of the modern-day butterflies, including the Hesperoidea and the Papilionoidea (Kristensen & Skalski, 1998). Although the bat-detecting hedylid ear differs in form from the Vogel's organ, it does appear to be homologous based on wing venation and innervation patterns of the forewing. Yack *et al.* (2000) suggest Vogel's organ may be a form of modification of the ancestral bat-detecting condition. It is possible that when butterflies became day active, some developed a sense of hearing in the low frequency range. Diurnality would have relieved the selection pressure imposed by bats, but introduced new selection pressures from diurnal predators. These new selection pressures may have resulted in the modification of bat-detecting ears to low-frequency hearing organs. This scenario implies that hearing would have been lost several times within the butterflies. Although complete loss of a tympanal organ in groups that are ancestrally tympanate is a rare phenomenon, there are Lepidopteran cases where this has occurred. For example, this has been reported in the diurnal lymnatriid *Penthophera morio* (Egger & Gohrbandt, 1938; Kiriakoff, 1950, both cit. in Minet & Surlykke, 2003). Thus, it is possible that a

sense of hearing has been lost several times in Nymphalidae groups including *Idea* and *Heliconius*. Perhaps more difficult to justify is a complete loss of hearing in all of the Hesperoidea. The possibility that Hedyloidea is more closely related to Nymphalidae than we once thought cannot be ruled out either. It is difficult to draw conclusions regarding the evolution of butterfly hearing without ample knowledge of an acoustic sense in butterflies – there are too many unknown cases at this point. The taxonomy of butterflies is highly debatable and further phylogenetic studies including morphological study of hearing organs and possibly life history aspects may aid in the resolution of butterfly groups. Resolving the question of the evolution of butterfly hearing requires more comparative analysis including anatomical, physiological, and behavioural facets.

4.4 Conclusions

Confirming a sense of hearing, and exploring the function of the Vogel's organ in *P. aegeria* sets the groundwork for future comparative studies of hearing in butterflies, particularly the Satyrinae. Results suggest that *Pararge aegeria* do possess a functional sense of hearing. Although further behavioural studies are required for confirmation, there is good evidence that the Vogel's organ of *P. aegeria* functions in the detection of avian predators. Comparative study of Vogel's organ in Satyrinae revealed that this character is found throughout the subfamily and is well-developed. Assembling more information on the life-history of these butterflies may provide explanation for the prominence of Vogel's organ in Satyrinae as well as the function of these ears. Within Nymphalidae, the Vogel's organ is widespread yet exhibits a high degree of variation. Further comparative study, including physiological and behavioural aspects, is likely to reveal a diversity of functions. The Vogel's organ is of great interest from a taxonomic

point of view based on its structural and functional complexity (this study, Otero, 1990a). Exploring the distribution and variation of this widespread character could potentially provide insight into the evolution of butterfly hearing and the phylogenetic relationships of Nymphalidae.

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Appendix I

Measurements of the forewing base, veins, and Vogel's organs (where applicable) in Satyrinae and related Nymphalidae outgroup specimens included in the comparative survey. Values given are average length or width of 2 (or 3) measurements taken per specimen. (AN, anal vein; Cu, cubital vein; Fw, forewing; IVO, inner Vogel's organ; OVO, outer Vogel's organ; SA, surface area; Sc, subcostal vein). All values are given in μm. Genera denoted with (OG) are the Nymphalidae outgroup representatives (i.e. non-Satyrinae).(i) Measurements of the forewing veins.(ii) Measurements of the forewing and Vogel's organ.

(i)

Genus	Sc Width	An Width	Cu width
Aphantopus	904.17	289.50	808.93
Brintesia	1446.94	388.15	929.05
Caligo (OG)	1561.99	802.56	1339.68
Cercyonis	1149.50	393.75	821.51
Charaxes (OG)	676.49	603.47	593.38
Cithaerias	1170.91	503.14	585.25
Coenonympha	535.44	444.59	572.58
Elymnias	924.23	362.35	414.97
Enodia	727.44	326.67	429.14
Erebia	756.32	311.14	378.35
Gnophodes	572.36	417.40	468.51
Heliconius (OG)	550.67	286.97	414.63
Heteronympha	909.91	669.42	1315.07
Hipparchia	1030.19	458.93	1000.71
Idea (OG)	502.77	337.64	303.25
Lasiommata	853.68	311.40	490.51
Manataria	652.22	442.83	577.77
Maniola	959.59	403.10	868.04
Melanargia	907.25	229.45	482.18
Melanitis	852.70	603.80	710.04
Morpho	895.98	1001.88	1482.86
Oeneis	750.74	251.13	387.81
Pararge	607.38	251.79	669.10
Pedaloides	1148.22	443.99	540.93
Pierella	1331.68	683.38	806.51
Satyrodes	785.65	350.89	571.68
Satyrus	1157.46	434.43	952.74
Stichophthalma	1018.36	670.85	638.29

(ii)

Genus	Fw Length	OVO Length	OVO Width	IVO Length	IVO Width	OVO SA	IVI SA
Aphantopus	22500	700.65	363.91	314.09	212.95	191816.99	59872.90
Brintesia	40300	1253.69	490.06	394.94	208.37	483464.30	70431.57
Caligo (OG)	75720	2409.69	1291.74	n/a	n/a	2136969.27	n/a
Cercyonis	29060	906.75	444.41	524.08	289.25	317544.61	121417.78
Charaxes (OG)	37700	1031.47	642.95	499.04	330.84	488840.75	118303.35
Cithaerias	32960	922.46	527.07	519.91	390.60	340867.74	145189.46
Coenonympha	15580	544.50	256.95	380.63	188.90	112963.40	56525.93
Elymnias	29520	884.63	416.75	524.84	259.44	270831.91	116929.34
Enodia	28141	719.15	396.48	425.21	270.86	203475.30	92631.15
Erebia	25600	794.60	436.12	490.92	271.12	245284.65	99572.78
Gnophodes	32800	951.71	380.15	214.90	173.27	256287.97	31685.73
Heliconius (OG)	46420	n/a	n/a	n/a	n/a	n/a	n/a
Heteronympha	29330	1068.38	503.56	575.25	314.26	400617.34	137470.67
Hipparchia	25280	934.68	381.08	635.43	227.95	268152.75	120490.19
Idea (OG)	60400	n/a	n/a	n/a	n/a	n/a	n/a
Lasiommata	23140	761.05	364.06	481.82	238.25	205024.71	85305.93
Manataria	36400	966.50	454.08	619.06	291.92	334229.50	152826.51
Maniola	25100	911.89	365.63	520.06	259.17	273448.29	106019.38
Melanargia	24300	760.10	345.25	427.12	312.73	200631.86	66746.31
Melanitis	42200	1144.61	573.12	517.52	263.25	483240.09	112392.71
Morpho	58900	1418.36	865.73	685.36	477.73	850347.88	245110.07
Oeneis	24460	756.74	403.08	454.71	215.83	219304.83	72198.71
Pararge	20590	645.08	289.76	375.36	180.33	145671.67	51670.76
Pedaloides	27500	880.79	415.11	553.55	277.78	268328.17	115120.05
Pierella	38940	1150.73	588.11	725.00	358.56	468882.10	192407.03
Satyrodes	30020	788.40	403.47	456.91	267.96	229342.41	101433.48
Satyrus	28630	1108.89	400.56	637.30	217.90	376654.18	116384.47
Stichophthalma	51540	1495.11	925.03	n/a	n/a	915762.71	n/a

Appendix II

Linear regressions used to examine the correlation between Vogel's organ dimensions and body size.