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Comparative anatomy of the caudal auricular muscles and their implications for the evolution of echolocation behavior in bats

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Abstract

Bats can be phylogenetically classified into three major groups: pteropodids, rhinolophoids, and yangochiropterans. While rhinolophoids and yangochiropterans are capable of laryngeal echolocation, pteropodids lack this ability. Delicate ear movements are essential for echolocation behavior in bats with laryngeal echolocation. Experimental evidence has shown that ear motions have an impact on echolocation ability in bats. Caudal auricular muscles, especially the cervicoauricularis group, play a critical role in such ear movements. Previously, caudal auricular muscles were studied in three species of bats with laryngeal echolocation. The cervicoauricularis group shows four muscle components in rhinolophoids, while three muscle components in yangochiropterans. However, to my knowledge, there have been no studies on non-laryngeal echolocators, the pteropodids. Therefore, this study fills in the gap of lacking muscle morphology and innervation data for the pteropodids. By comparing three major groups of bat data, I used the perspective of ear muscle morphology to understand the evolution of bat echolocation.

In Chapter 1, I described the gross anatomy of the cervicoauricularis muscles in *Cynopterus sphinx* by using diffusible iodine-based contrast-enhanced computed tomography (diceCT) and their innervation by 3D reconstructions of immunohistochemically stained serial

fetus sections. A previous study on bats with laryngeal echolocation reported that rhinolophoids have four cervicoauricularis muscles and yangochiropterans have three. I observed three cervicoauricularis muscles in the pteropodid *C. sphinx*. The number of cervicoauricularis muscles and their innervation pattern were comparable to those of non-bat boreoeutherian mammals and yangochiropterans, suggesting that pteropodids and yangochiropterans maintain the general condition of boreoeutherian mammals and that rhinolophoids have a derived condition. The unique nomenclature had been previously applied to the cervicoauricularis muscles of bats with laryngeal echolocation, but given the commonality between non-bat laurasiatherians and bats, with the exception of rhinolophoids, maintaining the conventional nomenclature (i.e., *M. cervicoauricularis superficialis*, *M. cervicoauricularis medius*, and *M. cervicoauricularis profundus*) is proposed for bats.

In Chapter 2, among bats, laryngeal echolocation is exhibited by rhinolophoids and yangochiropterans but not by pteropodids. Rousettus was regarded as the only pteropodid capable of echolocation using tongue clicks; however, growing evidence suggests that many species of pteropodids are capable of echolocation using wing clicks. Studies on laryngeal echolocators suggested that delicate ear movements are essential for echolocation behavior in bats, and cervicoauricularis muscles play a critical role in such ear movements. Here, I observed the gross anatomy of cervicoauricularis muscles in three species of pteropodids (*Cynopterus*

sphinx, *Eonycteris spelaea*, and *Rousettus leschenaultii*) to examine whether ear muscle anatomy varies among pteropodids with different echolocation types and differs between pteropodids and laryngeal echolocating bats. I found that the *M. cervicoauricularis profundus* originates from the nuchal crest in the tongue-click echolocator (*R. leschenaultii*) and from the midline in the wing-click echolocators (*C. sphinx* and *E. spelaea*). In general, tongue-click echolocation using high click rates is regarded as more sophisticated in sonar performance than wing-click echolocation. The *M. cervicoauricularis profundus* originating from the nuchal crest (CPNC) is not common among non-bat laurasiatherian mammals but can be found in laryngeal echolocating bats. Given that it helps pull the ear pinna caudally in the horizontal plane and increases the access to sound information, the CPNC found in *R. leschenaultii* and laryngeal echolocating bats may be a key characteristic for the sophisticated active echolocation behavior in bats.

The cervicoauricularis group is important because it shows various forms with different echolocation behaviors in bats. Primitive wing-click echolocators (*C. sphinx*) show three-muscle components closer to that of other non-bat laurasiatherians. In tongue-click echolocator (*R. leschenaultii*) also has three-muscle components but with CPNC features. In laryngeal echolocator of yangochiropterans (*Myotis myotis*) has three-muscle components with CPNC features. In the most complex laryngeal echolocator, rhinolophoids have four-muscle components with CPNC features. Therefore, I suspect that the number of components in

cervicoauricularis muscles and the location of the muscle origin may be evolutionarily linked to echolocation function in bats. This study proposed the nomenclature of muscles maintaining the conventional nomenclature and the diversity of the cervicoauricularis group in pteropodids. The results provide a foundation for future research on ear muscle studies in bats. Further studies on ear muscles in various bats are expected to shed further light on the function, homology, and evolution of these muscles in bats.

Keywords: comparative anatomy, echolocation, evolution, skull
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General introduction

Phylogenetic classification of bats and their characteristics

Bats (Chiroptera) are the second-largest group of mammals in the world, with approximately 1,400 species and globally distributed (Simmons & Cirranello, 2020; Zachos, 2019). Their distribution represents one of the greatest adaptive radiations in the history of mammals. This is largely due to their laryngeal echolocation and powered flight capabilities, which have enabled them to conquer the night sky, a vast and hitherto unoccupied ecological niche (Teeling, 2009). Bats were formerly grouped in the superorder Archonta, along with the treeshrews (Scandentia), colugos (Dermoptera), and primates (Van Den Bussche & Hoofer, 2004). Modern genetic evidence now places bats in the magnorder Boreoeutheria, and superorder Laurasiatheria, which includes eulipotyphlans, carnivorans, pangolins, perissodactyls, cetartiodactyls (Murphy et al., 2001). Traditionally, Chiroptera was classified into two suborders, Megachiroptera (non-laryngeal echolocation) and Microchiroptera (laryngeal echolocation), based on their morphological and behavioral characteristics (K. E. Jones et al., 2002; Smith, 1976). However, based on molecular evidence, bats have been classified as Yinpterochiroptera and Yangochiroptera (Springer, 2013). The pteropodids, the only group among bats that lacks the ability of

laryngeal echolocation, are placed with the highly specialized echolocation rhinolophoids. The remain groups are placed into the Yangochiroptera. Before the advancement of molecular phylogeny and based on morphological traits, laryngeal echolocators were once considered to form a monophyletic group. However, the revision of the phylogenetic relationships within bats, led by molecular studies (Teeling et al., 2005; Tsagkogeorga et al., 2013), rekindled the debate on the evolutionary scenario under which laryngeal echolocation arose. Therefore, how echolocation and flight originated in bats remain unanswered and has been highly debated (Davies et al., 2013; Nojiri et al., 2021; Nancy B. Simmons, 2005; Teeling et al., 2016; Veselka et al., 2010; Wang et al., 2017).

Echolocation of bats

The avoidance of obstacles by flying bats was raised by Spallanzani (1794) (G. Jones, 2005). The perception of bats in invisible conditions had been a mystery. The term echolocation was mentioned by the American zoologist Donald Griffin first demonstrated the phenomenon in bats (Griffin, 1958). Echolocation is a biological sonar system accomplished by the production and reception of high-frequency sound, allowing animals to perceive the surrounding environment (Thomas et al., 2004). The echolocation system of bats consists of three major systems: vocalization, audition, and orientation (Jen, 1982). Generally, in laryngeal echolocating

bats, echolocation is commonly categorized by the vocalization system. The classification is based on the frequency spectrogram of the emitted calls. One recent attempt to categorize bat echolocation calls considered two different types of frequency structure: frequency modulated (FM) sweeps, and constant frequency (CF) with Doppler-shift compensation (DSC) (Fenton et al., 2012; G. Jones & Teeling, 2006; Schnitzler et al., 2003). FM echolocating bats emit short-duration, broadband, downward frequency modulated (FM) signals separated by relatively long periods of silence. In contrast, CF echolocating bats emit long-duration, narrowband calls dominated by a single constant frequency (CF) separated by relatively short periods of silence (Fenton et al., 2012). On the other hand, in pteropodids which be recognized as non-echolocating groups, some of the species utilize primitive echolocation instead of laryngeal echolocation. Primitive echolocation is commonly categorized by the method of creating the voice. The primitive echolocating species represented by pteropodids is the genus Rousettus, which can use primitive echolocation based on “tongue clicks” to navigate in caves (Holland & Waters, 2005; G. Jones & Teeling, 2006; Yovel et al., 2011). Growing evidence suggests that bats of non-lingual echolocating pteropodids, such as *Eonycteris*, *Cynopterus*, and *Macroglossus*, can also use a primitive echolocation form based on wing clicks (Boonman et al., 2014; Gould, 1988). Most pteropodids have an effective vision for orientation at night and have a reflective tapetum lucidum to enhance visual sensitivity at low light levels (Ollivier et al.,

2004). Being frugivorous and nectarivorous, pteropodids do not need to use high-frequency echolocation calls to detect small targets such as insects. However, their primitive echolocation ability makes them more suitable for living at night and cave dwellings. Echolocation, similar to morphology, is a flexible character that is often shaped more by ecological demands than by phylogeny. Although echolocation provides some remarkable examples of convergent evolution within bats, this same convergence makes the reconstruction of ancestral call types problematic (G. Jones & Teeling, 2006).

Caudal auricular muscles

Auricularis muscles are a group of small muscles located in the external ear (pinna) that are responsible for moving the ears in various directions (Evans & de Lahunta, 2013). Based on their relative positions to the ear pinnae the muscles can be classified into four groups, dorsal *(M. scutuloauricularis*), ventral (*M. mandibuloauricularis, M. zygomaticoauricularis*), rostral (*M. adductor auricularis, M. zygomaticoauricularis*) and caudal (*M. cervicoauricularis, M. occipitalisauricularis, M. cervicoscutularis*). The function of each group is to control the movement of the ear cartilage in the direction corresponding to the group. Among these ear-muscle groups, the caudal auricular muscle group has the largest number of muscles and shows the highest diversity of morphology. However, rhinolophids and hipposiderids have highly differentiated caudal auricular muscles and can create Doppler shift by alternating ear motions with one ear moving forward while the other moves backward at high speed (Yin & Müller, 2019; Yin et al., 2017). Through fast ear motions, bats can encode information on target direction into time frequency Doppler signatures (Yin & Müller, 2019; Yin et al., 2017). Because of this function, I was curious about the evolution of the caudal auricular muscles. Where did this particular muscle group evolve in bats, how did this rapid ear movement behavior evolve, and what is the difference

in muscle compared to other bats? The caudal auricular muscles, innervated by the facial nerve, generally originate from the dorsal cervical region and insert into the auricle in mammals (Budras et al., 2013, 2012; Evans & de Lahunta, 2013). To date, caudal auricular muscles have been studied for two rhinolophoids (*Rhinolophus ferrumequinum* and *Aselliatridens*) (Schneider, 1960; Schneider & Möhres, 1960) and one yangochiropteran (*Myotis myotis*) (Schneider, 1960), but there have been no reports on pteropodids.

Purpose of this study

The evolution of echolocation ability in bats has been a highly debated issue to investigate. In this study, I explored the evolution of bat echolocation from the perspective of outer ear movements using the auricularis muscles. Among three groups of bats, pteropodids, rhinolophoids and yangochiropteran, previous literature on bat ear muscles revealed four components of *M. cervicoauricularis* in rhinolophoids (Schneider, 1960; Schneider & Möhres, 1960) and three components of *M. cervicoauricularis* in yangochiropteran (Schneider, 1960). However, the remaining group, pteropodids were lacking studies. To further understand the evolutionary process of bat ear muscles, I describe in detail the cervicoauricularis muscles in the pteropodids and compare them to previous studies of muscle morphology. By comparing the ear muscle types among the three major groups of bats, I can identify the characteristic features of ear muscle types

associated with different echolocation behaviors. Through homology tracing, I can also infer the developmental origin of the additional ear muscles in rhinolophoids. Different morphology of muscles reflects different echolocation behaviors. Therefore, I can use this to deduce evolutionary information. By complementing the data of pteropodids, I can gain more insight into the overall evolution of bats.

Chapter 1 Anatomy and homology of the caudal auricular muscles in greater short-nosed fruit bat (*Cynopterus sphinx*)

1.1 Introduction

Bats (order Chiroptera) have undergone one of the greatest adaptive radiations in mammalian history, driven by the acquisition of powered flight and echolocation ability (G. Jones & Teeling, 2006). In the present account, three major groups, the pteropodids, rhinolophoids, and yangochiropterans, comprise the extant bats (Teeling et al., 2016). Among these, rhinolophoids and yangochiropterans are capable of laryngeal echolocation, while pteropodids lack the such ability (Griffin, 1958; Yovel et al., 2010). Although pteropodids are not equipped with the sophisticated biosonar ability to perform laryngeal echolocation, many of them are able to conduct primitive types of echolocation such as tongue-click echolocation and wing-click echolocation (Boonman et al., 2014). Echolocation strategies are also highly diverse among bats with laryngeal echolocation (G. Jones & Teeling, 2006).

Experimental evidence has shown that ear motions have an impact on echolocation ability in bats (Mogdans et al., 1988; Möhres & Kulzer, 1955, 1956; Schneider & Möhres, 1960; Yin et al., 2017). Rhinolophids and hipposiderids actively create Doppler shifts with their fast-moving pinnae to encode additional sensory information (Yin & Müller, 2019). The caudal auricular muscles play a critical role in triggering ear movement, and inhibition of the caudal auricular muscles is reported to decrease echolocation performance (Müller, 2015; Yin & Müller, 2019). However,

the diversity of ear movements and their link to the variation in echolocation behavior in bats remains largely unclear.

To date, caudal auricular muscles have been studied for two rhinolophoids (*Rhinolophus ferrumequinum* and *Asellia tridens*) (Schneider, 1960; Schneider & Möhres, 1960) and one yangochiropteran (*Myotis myotis*) (Schneider, 1960), but there have been no reports on pteropodids. Here, I describe the caudal auricular muscles and their innervation pattern in the greater short-nosed fruit bat (*Cynopterus sphinx*). To our knowledge, this is the first report of its kind for pteropodids. *C. sphinx* is widely distributed from Pakistan, India, and Sri Lanka to southern China and Vietnam, the Malay Peninsula, Sumatra, Java, Borneo, Sulawesi, Timor, and smaller islands in the Malay Archipelago (Bates & Harrison, 1998; Boonsong & McNeely, 1977; Corbet & Hill, 1992; Kitchener, 1991). It is common in tropical forests and areas where fruit crops are cultivated (Srinivasulu & Srinivasulu, 2002). I first examined the number of muscles, attachments, and innervation of the caudal auricular muscles and then compared them with those of other bats and non-bat mammals. This study resolves the confused nomenclature for the caudal auricular muscles and provides a unifying framework to understand the evolution and variation of the caudal auricular muscles in bats.

1.2 Materials and methods

Adult *C. sphinx* obtained from the collection of the Institute of Ecology and Biological Resources, the Vietnam Academy of Science and Technology (IEBR; Ha Noi, Vietnam) were fixed and preserved in 70% ethanol. To enhance the contrast of the CT images, I followed the image enhancement techniques used in a previous study (diceCT) (Gignac et al., 2016) and stained the specimen with 1% iodine in 100% ethanol for 14 days before scanning. The specimen was observed macroscopically and then scanned using a microCT system (inspeXio SMX-90CT Plus, Shimadzu Corp., Kyoto, Japan) with 90KV source voltage and 100 μA source current; the voxel size of the images was 39 μm. I reconstructed the microCT scan of an adult fruit bat in the cranial part using Amira 5.2 software (Visage Imaging, San Diego, USA).

A late-stage fetus (Stage 22) of *C. sphinx* was used for serial sectioning and 3D reconstructions. Staging was conducted based on the embryonic development of the Egyptian fruit bat(*Rousettus aegyptiacus*) (Khannoon et al., 2019). The cranium of the fetal sample was dehydrated with a graded series of ethanol (70–100%), cleared in xylene, and embedded in paraffin. The embedded sample was cut into 9 μm-thick sections using a rotary microtome HM355S and Section Transfer System (Thermo Fisher, MA, USA) and then placed on glass slides (CRE-04, Matsunami Glass Ind., Osaka, Japan). Deparaffinized sections were stained with hematoxylin and

eosin (H & E) (Sakura Finetek, Tokyo, Japan) and underwent immunohistochemical staining with a mouse anti-acetylated tubulin monoclonal antibody (1:2000, Sigma-Aldrich, no. T7451). The staining was processed with biotinylated anti-mouse IgG (1:200, BA-2000, Vector Laboratories Inc., CA, USA) followed by Avidin–Biotin Complex (Vectastain ABC Kit, Peroxidase (standard) PK-4000, Vector Laboratories) and Takara DAB Substrate (MK210, Takara Bio, Shiga, Japan). After staining, the sections were dehydrated in a graded series of ethanol, cleared in xylene, and covered with cover glass (NEO, Matsunami Glass Ind., Osaka, Japan). Images of serial sections were captured using a light microscope (SMZ-18, Nikon Corp., Tokyo, Japan) equipped with a digital camera (DS-Ri2, Nikon Corp., Tokyo, Japan) connected to a computer. The 3D reconstruction was conducted using Avizo 6.3 (Visualization Sciences Group, Hillsboro, USA).

An embryonic day 14.5 fetus (E14.5) of wild-type *Mus musculus* (C57BL/6J) was studied as an outgroup. The embryo was fixed in modified Serra's fixative (4% PFA containing ethanol and acetic acid), dehydrated, and embedded in paraffin wax. Sections were cut at a thickness of 6 μm. I used anti-acetylated tubulin (1:2000, Sigma-Aldrich, no. T7451) and HRP-conjugated polyclonal goat anti-mouse (no. P0447, Dako, Glostrup, Denmark) to visualize the nerve axons. Next, the sections were stained with Alcian blue and H & E, following standard protocols. Images of serial sections were captured using a light microscope (BX60, Olympus Corp.,

Tokyo, Japan) equipped with a digital camera (DP70, Olympus Corp., Tokyo, Japan) connected to a computer. The 3D reconstruction was performed using Amira 2022.2.

1.3 Results

The 3D reconstruction from the diceCT scan of the adult *C. sphinx* (Fig. 1A, 1B) clarified the shape and topology of the caudal auricular muscles and occipitopollicalis muscle. The most rostral muscle originated from the midline of the skull and inserted into the upper part of the auricle (Fig. 1B). The second muscle originated from the midline of the skull but ran underneath the most rostral muscle and inserted into the middle level of the auricle (Fig. 1B). The third muscle, located most rostrally among the three muscles, originated from the midline of the skull and inserted into the lower part of the auricle (Fig. 1B). A wing-related muscle, the occipitopollicalis, which is peculiar to bats, was a very long superficial muscle that originated from the caudal midline region of the skull and inserted into the propatagium (Fig. 1B).

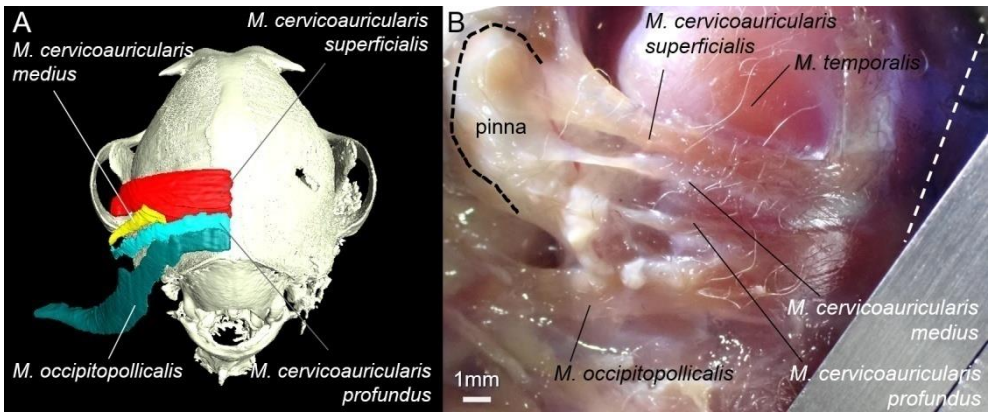

Fig. 1 Cervicoauricularis muscles in the adult *Cynopterus sphinx*.

(A) 3D reconstruction of adult *C. sphinx* head in caudal view. (B) Gross dissection of the temporal region in dorsal view. The dashed white line indicates the midline of the skull.

The 3D reconstruction of serial sections from the fetal specimen clarified the shape topology and innervation of the caudal auricular muscles, *M. occipitopollicalis*, and the nearby facial muscles such as the platysma (Fig. 2A). All three caudal auricular muscles and *M. occipitopollicalis* were present at this stage, already showing the basic topological patterns observed in the adult specimen (Figs. 1, 2). The platysma was a very thin but wide superficial muscle in *C. sphinx* adults, but in the fetal specimen, it was smaller, and only located laterally to the mandible. In this fetal specimen, I was able to observe the peripheral morphology of the facial nerve (Fig. 2A, 2B). The facial nerve had five branches (Fig. 2B). The *N. retroauricularis* supplied all three caudal auricular muscles. The *M. occipitopollicalis* was supplied by the *N. retroauricularis inferior* (Schneider, 1960; Schneider & Möhres, 1960), which was the first subbranch from the *N. retro auricularis*. The platysma was found to be supplied by the *ramus colli*. The *N. digastricus*, which supplied the posterior belly of the digastric muscle, and the *ramus buccalis ventralis*, which was the thickest branch extending to the buccal nerve and mandibular nerve, were also identified (Fig. 2B).

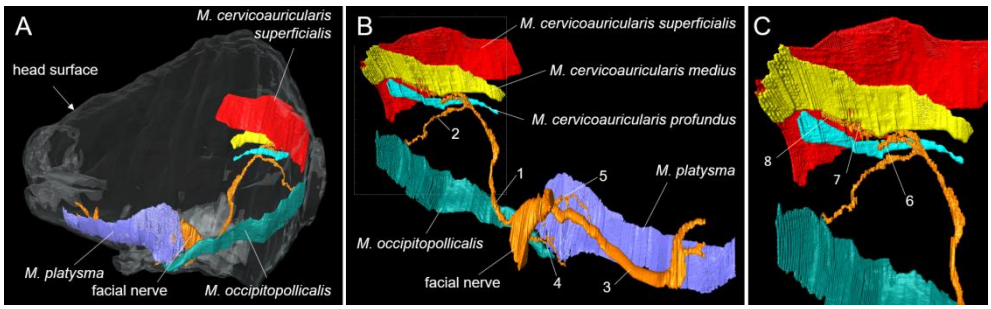

Fig. 2 3D reconstructions of Stage 22 fetal *Cynopterus sphinx* head.

(A) Cervicoauricularis muscles and neighboring muscles in left lateral view. (B) Medial view of the left side head. (C) Higher magnification of the dashed region in B. 1: *N. retroauricularis*, 2: *N. retroauricularis inferior*, 3: *N. facialis ramus buccalisventralis*, 4: *N. digastricus*, 5: *N. facialis ramus colli*, 6: *N. retroauricularis ramus cervicoauricularis profundus*, 7: *N. retroauricularis ramus cervicoauricularis medius*, 8: *N. retroauricularis ramus cervicoauricularis superficialis*.

The 3D reconstruction of serial sections from the E14.5 fetal *M. musculus* specimen clarified the shape, topology, and innervation of the caudal auricular muscles (Fig. 3A). All three caudal auricular muscles were present at this stage. In this fetal specimen, I was able to observe the peripheral morphology of the facial nerve. The *N. retroauricularis* supplied all three caudal auricular muscles (*cervicoauricularis superficialis*, *cervicoauricularis medius*, and *cervicoauricularis profundus*), as shown in Figure 3B. The *cervicoauricularis medius* was found to run under the *cervicoauricularis superficialis* and *profundus*. The insertion of the *cervicoauricularis medius* was found to be ventral to the *cervicoauricularis superficialis*. The insertion of *cervicoauricularis superficialis* was found to be the most rostral among the three muscles. The insertion of *cervicoauricularis profundus* was found to be the most caudal among the three muscles.

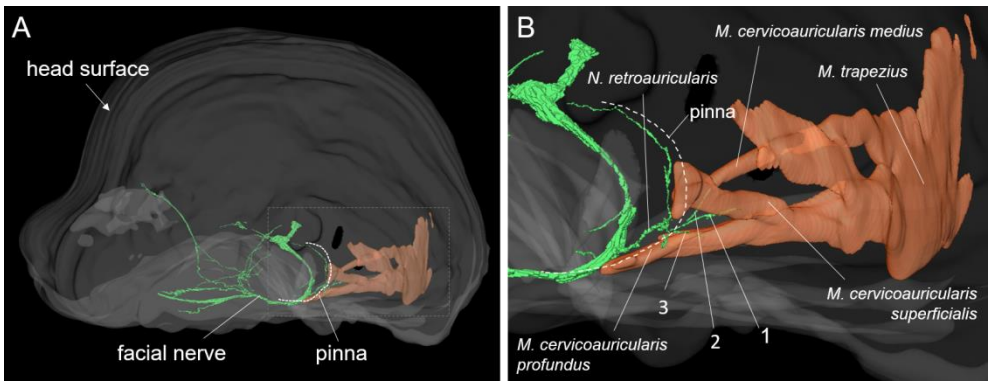

Fig. 3 3D reconstructions of E14.5 fetal *Mus musculus* head in left lateral view.

(A) 3D reconstruction in lateral view. The facial nerve and its branches are given in green and muscles in the retroauricular region are given in red. The position of the pinna is indicated by the dashed white line. (B) Higher magnification of the dashed region. The position of the auricle is indicated by the dashed white line. 1: *N. retroauricularis ramus cervicoauricularis superficialis*, 2: *N. retroauricularis ramus cervicoauricularis medius*, 3: *N. retroauricularis ramus cervicoauricularis profundus*.

1.4 Discussion

The results showed three components of the cervicoauricularis muscles in *C. sphinx*, originating from the midline of the skull (Fig. 1). Previous studies by Schneider (Schneider, 1960; Schneider & Möhres, 1960) reported that the laryngeal echolocating rhinolophoids have different numbers of cervicoauricular muscle components compared to non-bat mammals. The most distinctive features of the muscles of the rhinolophoids are that the cervicoauricularis has four components and that the two caudal muscles, the *cervicoauricularis profundus major*, and *minor*, do not originate from the midline but from the nuchal crest. In the horseshoe bat (*R. ferrumequinum*), in addition to the *cervicoauricularis profundus major* and *profundus minor*, the *cervicoauricularis superficialis minor* also originates from the nuchal crest. Its most rostral cervicoauricularis muscle, the *cervicoauricularis superficialis major*, originates from the midline. In the trident bat (*A. tridens*), the *cervicoauricularis superficialis major* and *minor* originate from the midline, and the *cervicoauricularis profundus major*and *minor* originate from the nuchal crest. The mouse-eared bat (*M. myotis*), which belongs to the Yangochiroptera, was reported by Schneider (Schneider, 1960) to have only three muscles (the *cervicoauricularis superficialis major*, *cervicoauricularis superficialis minor*, and *cervicoauricularis profundus minor*) (but note that inconsistent muscle names are used between his figures and some parts of the text, which is

possibly merely a typo). In this species, the two rostral muscles, the *cervicoauricularis superficialis major* and *cervicoauricularis superficialis minor*, originate from the midline, whereas the most caudal *cervicoauricularis profundus minor* originates from the nuchal crest (Schneider, 1960; Schneider & Möhres, 1960). While the most caudal or two caudal cervicoauricularis muscles originate from the nuchal crest instead of the skull midline in the three laryngeal echolocating bats (*R. ferrumequinum*, *A. tridens*, and *M. myotis*) (Schneider, 1960; Schneider & Möhres, 1960), all three muscles originate from the midline in *C. sphinx* (Fig. 1).

Phylogenetically, the yinpterochiropterans *C. sphinx* is more closely related to *R. ferrumequinum* and *A. tridens* than to the yangochiropteran *M. myotis*, but *R. ferrumequinum*, *A. tridens*, and *M. myotis* are capable of laryngeal echolocation while *C. sphinx* lacks such ability. Whether *C. sphinx* or laryngeal echolocating bats retains the ancestral condition for ear muscle attachment pattern can be inferred by comparing them with other non-bat laurasiatherians. Terrestrial eulipotyphlans (the common shrew *Sorex araneus* and European hedgehog *Erinaceus europaeus*) have three components of the cervicoauricularis (Burda, 1979; Meinertz, 1978). The origin of the muscles is also from the midline; however, the origin is not from the skull as in bats but from the midline of the dorsal side of the neck (Burda, 1979; Meinertz, 1978). Another eulipotyphlan, the hairy-tailed mole (*Parascalopsbreweri*), lacks

the pinna but possesses two cervicoauricularis muscles. The *superficialis* originates from the dorsal midline of the neck and inserts into the ear canal, while the *profundus* originates under the *superficialis* from the midline and inserts into the forelimb (Whidden, 2000). These reports suggest that the cervicoauricularis muscles commonly originate from the dorsal midline of the neck in eulipotyphlans. In carnivorans, felids (domestic cat *Felis catus*, tiger *Panthera tigris*, and serval *Leptailurus serval*) show a cervicoauricularis muscle pattern that is comparable to that of *C. sphinx*, with the same three muscles originating from the caudal region of the midline of the skull (Diogo et al., 2012; Reighard & Jennings, 1901). The dog (*Canis lupus*) also has three muscle components of the cervicoauricularis muscles. The cervicoauricular muscles do not originate from the skull but from the nuchal ligament itself (Evans & de Lahunta, 2013; Heine, 2004; Huber, 1918). The nuchal ligament is located in the midline; thus, *C. lupus* still follows the "midline rule" for cervicoauricularis muscle origins. Perissodactyls (*Equus caballus* and *Tapirus terrestris*) have three components of the cervicoauricularis muscles, all of which originate from the insertion site of the nuchal ligament that is again in the midline (Budras et al., 2012; Yaşar, 1977).A member of the cetartiodactyls, the cow (*Bos taurus*), shows a comparable number and pattern of origin to those of perissodactyls (Budras et al., 2013).

Taken these together, the ancestral laurasiatherian condition of muscle number of the cervicoauricularis muscle is postulated to be three.

The number of cervicoauricularis muscles of laurasiatherians is summarized in Table 1. The three-muscle pattern seems to be widely conserved among mammals, and the two-muscle condition found in the talpids is likely to be a derived condition. I suggest that *C. sphinx* retains the ancestral condition of laurasiatherians, the three-muscle condition, and that the four-muscle condition found in rhinolophoids is a derived condition for laurasiatherian mammals. The wide survey of laurasiatherians also suggests that all of them follow the "midline rule" for the origin of the cervicoauricularis, although the origin site may vary from the skull or neck.

Table 1

Order	Species	Number of cervicoauricularis muscle
Eulipotyphla	*Sorex Araneus* (Burda, 1979)	3
	Erinaceus europaeus (Meinertz, 1978)	3
	Parascalops breweri (Whidden, 2000)	2
Chiroptera	*Rhinolophus ferrumequinum* (Schneider & Möhres, 1960)	4
	Asellia tridens (Schneider, 1960)	4
	Myotis myotis (Schneider, 1960)	3
Carnivora	*Felis catus* (Reighard & Jennings, 1901)	3
	Leptailurus serval (Diogo et al., 2012)	3
	Panthera tigris (Diogo et al., 2012)	3
	Canis lupus (Evans & de Lahunta, 2013; Heine, 2004; Huber, 1918)	3
Perissodactyla	*Equus caballus*(Budras et al., 2012)	3
	Tapirus terrestris (Yaşar, 1977)	3
Cetartiodactyla	*Bos taurus* (Budras et al., 2013)	3

The observations on fetal anatomy allowed me to address the homologies of the cervicoauricularis muscles among mammals. Detailed information on cervicoauricularis innervation is still largely lacking, but in *C. lupus*(Evans & de Lahunta, 2013; Heine, 2004; Huber, 1918), *E. caballus* (Budras et al., 2012), *B. taurus* (Budras et al., 2013), and *M. musculus*, all cervicoauricularis muscles (*superficialis*, *medius*, and *profundus*) are innervated by the *N.* retroauricularis (Budras et al., 2013, 2012; Evans & de Lahunta, 2013). The E14.5 fetus of *M. musculus* in this study showed three components of the cervicoauricularis muscles, which agreed with observations on various rodents (*Laonaste saenigmamus*, *Ctenodactylus gundi*, and *Chinchilla lanigera*) by Zherebtsova (Zherebtsova, 2012). According to Zherebtsova, in adult rodents, the *cervicoauricularis superficialis* is dorsal to the *cervicoauricularis medius*, and its insertion is the most rostral among the three muscles. The insertion of the *cervicoauricularis profundus* is the most caudal among the three muscles. The insertion of the *cervicoauricularis medius* is located between those of the *cervicoauricularis superficialis* and *cervicoauricularis profundus*. The origins of all three muscles are from the midline. In the E14.5 fetus, the muscles were not fully developed; thus, their origins were not yet from the midline, but the relative topologies of the three muscles were comparable to those reported by Zherebtsova and also to those of *C. sphinx* (in this study) and *M. myotis* (Schneider, 1960). The observation of the fetus revealed that

all three cervicoauricularis muscles of *C. sphinx* are innervated by the *N. retroauricularis*. Conventionally, there has been no specific nomenclature for the subbranches of *N. retroauricularis* that innervate the three cervicoauricularis muscles; therefore, I term the subbranch of *N. retroauricularis* innervating the *cervicoauricularis superficialis* as the *ramus cervicoauricularis superficialis*, the subbranch innervating the *cervicoauricularis medius* as the *ramus cervicoauricularis medius*, and the subbranch innervating the *cervicoauricularis profundus* as the *ramus cervicoauricularis profundus*, respectively (Fig. 3). Given that the branch innervating the occipitopollicalis (*R. ferrumequinum*, *A. tridens*, and *M. myotis*) was referred to as the *N. retroauricularis inferior* by Schneider (Schneider, 1960; Schneider & Möhres, 1960), I term the other branch innervating the *cervicoauricularis superficialis*, *cervicoauricularis medius*, and *cervicoauricularis profundus* as the *N. retroauricularis superior.* To my knowledge, the relative topologies of the rami of *N. retroauricularis* innervating the three muscles are largely undescribed even among domestic animals; thus, available references are poor, but my detailed observations found that the innervation pattern of the three cervicoauricularis muscles in *M. musculus* is comparable to those in *C. sphinx* and *M. myotis* (Fig. 4). Given the homogeneity of the cervicoauricularis muscle numbers and innervation patterns among the three species, the three muscles in *C. sphinx* should be considered as the *cervicoauricularis superficialis*, *cervicoauricularis medius*, and *cervicoauricularis profundus*, as in *M.*

musculus (Fig. 3B). Schneider referred to the three muscles in *M. myotis* as the "*cervicoauricularis superficialis major*," "*cervicoauricularis superficialis minor*," and "*cervicoauricularis profundus minor*," but based on the homological examination, I propose to rather use "*cervicoauricularis superficialis*," "*cervicoauricularis medius*," and "*cervicoauricularis profundus*," respectively, following the nomenclature widely used in other mammals.

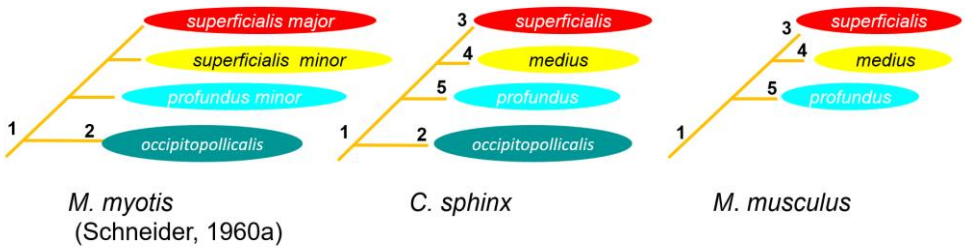

Fig. 4 Innervation patterns to the cervicoauricularis muscles in *Myotis myotis*, *Cynopterus sphinx*, and *Mus musculus*.

Schneider (Schneider, 1960) used different nomemclature, but muscle names in bats can follow the conventional nomenclature used for other mammals. 1: *N. retroauricularis*, 2: *N. retroauricularis inferior*, 3: *N. retroauricularis ramus cervicoauricularis superficialis*, 4: *N. retroauricularis ramus cervicoauricularis medius*, 5: *N. retroauricularis ramus cervicoauricularis profundus*.

I found that the muscle number and the three-muscle pattern of the cervicoauricularis is widely conserved among therian mammals. Innervation patterns are comparable between *C. sphinx*, *M. myotis* and *M. musculus* (Fig. 4), but ambiguity remains for the rhinolophoids (*R. ferrumequinum* and *A. tridens*), which have four cervicoauricular muscles (*cervicoauricularis superficialis major*, *cervicoauricularis superficialis minor*, *cervicoauricularis profundus minor*, and *cervicoauricularis profundus major*) (Schneider, 1960; Schneider & Möhres, 1960). Innervation to the "*cervicoauricularis superficialis major*," "*cervicoauricularis superficialis minor*," and "*cervicoauricularis profundus minor*" (Schneider, 1960; Schneider & Möhres, 1960) appears to be comparable to the *cervicoauricularis superficialis*, *cervicoauricularis medius*, and *cervicoauricularis profundus* in *C. sphinx*, *M. myotis*, and *M. musculus*, respectively; therefore, these should be treated as homologous. I postulate that the "*cervicoauricularis profundus major*" of rhinolophoids, which is innervated by the *N. retroauricularis inferior*, should be further studied and renamed in the future. Rhinolophoids that conduct constant-frequency echolocation show conspicuous pinna motions as part of their biosonar behavior, which are not found in frequency-modulated echolocating bats (the majority of yangochiropterans) or non-laryngeal echolocating bats (all pteropodids) (Yin et al., 2017). It is possible that the greater number of cervicoauricularis muscles reflects their rapid and delicate pinna motions. In addition, the most caudal or two caudal cervicoauricularis muscles originate

from the nuchal crest in laryngeal echolocators (*R. ferrumequinum*, *A. tridens*, and *M. myotis*), whereas all three muscles originate from the midline in terrestrial non-bat Placentals and in *C. sphinx*. Although not much is known about the functional significance of the varying muscle origins, perhaps there is some unknown link between the origin topology of the *cervicoauricularis profundus* and echolocation function. Further studies on ear muscles in various bats are expected to shed further light on the function, homology, and evolution of these muscles in bats.

Chapter 2 Caudal auricular muscle variation in pteropodid bats and implications for the evolution of echolocation behavior

2.1 Introduction

Extant bat species have been behaviorally categorized into two major types: 1) echolocating species that include members of the suborder Yangochiroptera (yangochiropterans) and the superfamily Rhinolophoidae (rhinolophoids) emitting calls produced by the larynx (laryngeal echolocators) and 2) non-echolocating species represented by family Pteropodidae (pteropodids) with the exception of genus *Rousettus*, which can use primitive echolocation based on "tongue clicks" (lingual echolocators) to navigate in caves (Holland & Waters, 2005; G. Jones & Teeling, 2006; Yovel et al., 2011). Growing evidence suggests that bats of non-lingual echolocating pteropodids, such as *Eonycteris*, *Cynopterus*, and *Macroglossus*, can also use a primitive echolocation form based on wing clicks (wing-click echolocators) (Boonman et al., 2014; Gould, 1988). The performance of echolocation (inferred from click rates) decreases from *Eonycteris* to *Cynopterus* and *Macroglossus* (Boonman et al., 2014), and the performance appears to be higher in cave dwellers (Boonman et al., 2014; Gould, 1988; Kulzer, 1958; Srinivasulu & Srinivasulu, 2002). Boonman et al. (Boonman et al., 2014) also found that *Rousettus aegyptiacus* uses wing clicks in addition to lingual echolocation. Therefore, these findings indicated that the evolutionary history of click-based primitive echolocation in pteropodids is complex; further examination of their genetic and morphological basis for echolocation is necessary to understand the origins

and evolution of echolocation in bats (Boonman et al., 2014).

Experimental evidence has shown that ear motions play a crucial role in bat echolocation behavior (Mogdans et al., 1988; Möhres & Kulzer, 1955; Schneider & Möhres, 1960; Yin et al., 2017) and differ considerably among bat species, possibly reflecting their divergent echolocation strategies. For instance, *Rhinolophus* (rhinolophoids) and *Hipposideros* (hipposiderids), which are members of rhinolophoids, actively create Doppler shifts with their fast-moving pinnae to encode additional sensory information (Yin & Müller, 2019). Their four cervicoauricularis muscles trigger ear movement and thus critically affect echolocation performance (Müller, 2015; Schneider & Möhres, 1960; Yin & Müller, 2019). Although pteropodids are now treated as echolocators, the role of ear movements and the basic anatomy of cervicoauricularis muscles in pteropodids remain largely unexplored. Chi et al. 2023 reported that the greater short-nosed fruit bat (*Cynopterus sphinx*) has three cervicoauricularis muscles similar to other laurasiatherian mammals but different from rhinolophoid bats with a four-muscle condition. Whether such divergences in cervicoauricularis muscles between rhinolophoids (laryngeal echolocators) and *Cynopterus* (non-laryngeal echolocator) are associated with different echolocation strategies and/or performances is unclear (Chi et al., 2023). Furthermore, how ear muscle anatomy varies among pteropodids with different echolocation performances is unknown.

Here, I comparatively studied the number of muscles and

attachments of cervicoauricularis muscles among three pteropodid species, namely, *C. sphinx*, *Eonycteris spelaea* (cave nectar bat), and *Rousettus leschenaultii* (Leschenault's rousette). *Cynopterus sphinx*is a frugivore common in tropical forests and areas where fruit crops are cultivated (Srinivasulu & Srinivasulu, 2002). *Rousettus leschenaultii*is a frugivorous cave dweller that uses tongue- and wing-click echolocation(Vanlalnghaka, 2015). *Eonycteris spelaea* is a nectarivorous cave dweller that uses wing-click echolocation and travels long distances to feed on nectar and fruits (Gould, 1988; Kitchener & Maharadatunkamsoi, 1990; Payne et al., 1998; Start & Marshall, 1976).In this work, I highlighted the diversity of muscle patterns in pteropodids with different echolocation types and discussed the possible link between variations in caudal auricular muscles and echolocation behavior.

2.2 Materials and methods

Chi et al. (Chi et al., 2023) previously reported the 3D reconstruction and macroscopic dissections of the cervicoauricularis muscles of *C. sphinx*. In the present study, I followed the muscle nomenclature proposed by Chi et al. (Chi et al., 2023). The *M. cervicoscutularis*, which attaches to the scutiform cartilage, was omitted from this study, given its minor role for ear movements in bats (Schneider, 1960; Schneider & Möhres, 1960). I studied the caudal auricular muscles that are directly attached to the caudal part of the pinna (*M. cervicoauricularis*) in the adult greater short-nosed fruit bat (*C. sphinx*), adult cave nectar bat (*E. spelaea*), and adult Leschenault's rousette (*R. leschenaultii*) that were curated at the collection of the Institute of Ecology and Biological Resources, the Vietnam Academy of Science and Technology (Hanoi, Vietnam). Bats were euthanized by 5% isoflurane inhalation and then fixed and preserved in 70% ethanol. One individual was used for scanning, and an additional sample was dissected and observed macroscopically for each species. The maturity of the samples was checked by the presence of fully erupted and occluded dentition. Image-enhancement techniques adopted from a previous study (Gignac et al., 2016) were used to visualize the muscular anatomy of the bats. Samples were stained with 1% iodine in 100% ethanol for 14 days followed by scanning using a micro computed tomography (microCT) system (inspeXio SMX-90CT Plus,

Shimadzu Corp., Kyoto, Japan) with a 90 KV source voltage and 100 uA source current. The voxel size of the images was 39 μm for *C. sphinx*, 18 μm for *E. spelaea*, and 20 μm for *R. leschenaultii*. I manually segmented and reconstructed the microCT scans using Amira 5.2 (Visage Imaging, San Diego, USA). Finally, I macroscopically dissected one additional adult sample each of *E. spelaea* and *R.leschenaultii,* fixed in Carnoy's solution, and preserved in 70% ethanolto confirm my 3D reconstructions.

2.3 Results

The 3D reconstructions from diceCT scans of *E. spelaea* and *R. leschenaultii* (Figs. 4, 5) clarified the shape and topology of the cervicoauricularis muscles. The 3D reconstruction of the adult *C. sphinx* previously reported by Chi et al. (Chi et al., 2023) is given in Figure 7 for comparison. The most rostral muscle originated from the midline of the skull and inserted into the upper part of the ear pinna (Fig. 7A, 7B). The second muscle originated from the midline of the skull, but it ran underneath the most rostral muscle and inserted into the middle level of the ear pinna (Fig. 7A, 7B). The third muscle, located most caudally among the three muscles, originated from the midline of the skull and inserted into the lower part of the ear pinna (Fig. 7A, 7B).

M. cervicoauricularis superficialis

Eonycteris ***s****pelaea* and *R. leschenaultia* had *M. cervicoauricularis superficialis* as the most rostral muscle that originated from the midline of the skull and inserted into the upper dorsal part of the ear pinna (Figs. 5A, 6A). On the other hand, the *M. cervicoauricularis superficialis* of *C. sphinx* was wider than those of *E. spelaea* and *R. leschenaultii*, and it originated from the *M. cervicoauricularis medius* and inserted into the upper dorsal part of the ear pinna (Fig. 7A).

M. cervicoauricularis medius

In *E. spelaea* and *R. leschenaultii*, the *M. cervicoauricularis medius* originated from the midline of the skull adjacent to the *M. cervicoauricularis superficialis* and inserted into the middle dorsal part of the ear pinna (Figs. 5A, 6A). In *C. sphinx*, the muscle originated from the midline of the skull underneath the *M. cervicoauricularis superficialis* (Fig. 5A).

M. cervicoauricularis profundus

In *E. spelaea*, *M. cervicoauricularis profundus*, the most caudal muscle, originated from the dorsal side that is slightly lateral from the midline of the skull adjacent to the *M. cervicoauricularis medius* and inserted to the lower medial part of the ear pinna (Figs. 5A, 8A). In *R. leschenaultii*, the muscle originated from the nuchal crest and inserted into the lower medial part of the ear pinna (Figs. 6A, 8B). In *C. sphinx*, the muscle originated from the midline of the skull adjacent to the *M. cervicoauricularis medius* and inserted into the dorsal lower part of the ear pinna (Figs. 7A, 8C).

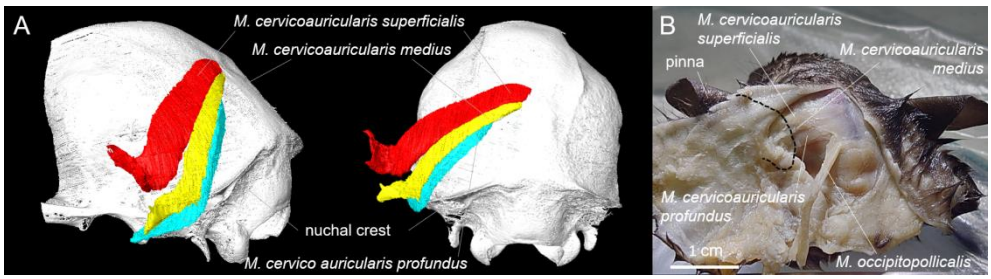

Fig. 5 Cervicoauricularis muscles and nearby structures in adult *Eonycteris spelaea*.

(A) Left lateral (left) and dorsal (right) views of the 3D reconstruction. (B) Gross dissection of the temporal region in dorsal view. Dotted line indicates the location of the covered pinna.

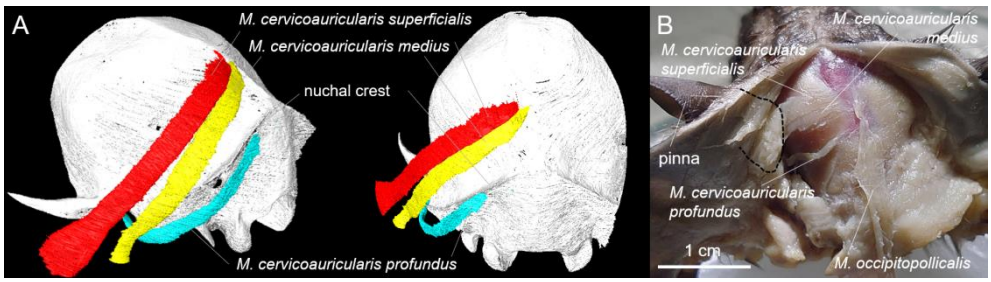

Fig. 6 Cervicoauricularis muscles and nearby structures in adult *Rousettus leschenaultii*.

(A) Left lateral (left) and dorsal (right) views of the 3D reconstruction. (B) Gross dissection of the temporal region in dorsal view. Dotted line indicates the location of the covered pinna.

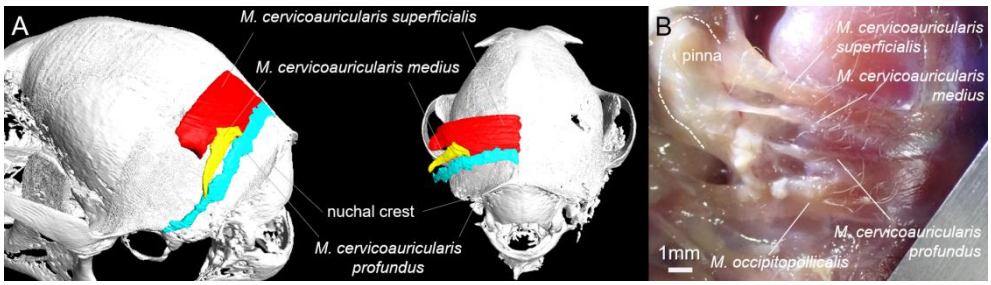

Fig. 7 Cervicoauricularis muscles and nearby structures in adult *Cynopterus sphinx*.

(A) Left lateral (left) and dorsal (right) views of the 3D reconstruction. (B) Gross dissection of the temporal region in dorsal view. Dotted line indicates the location of the covered pinna.

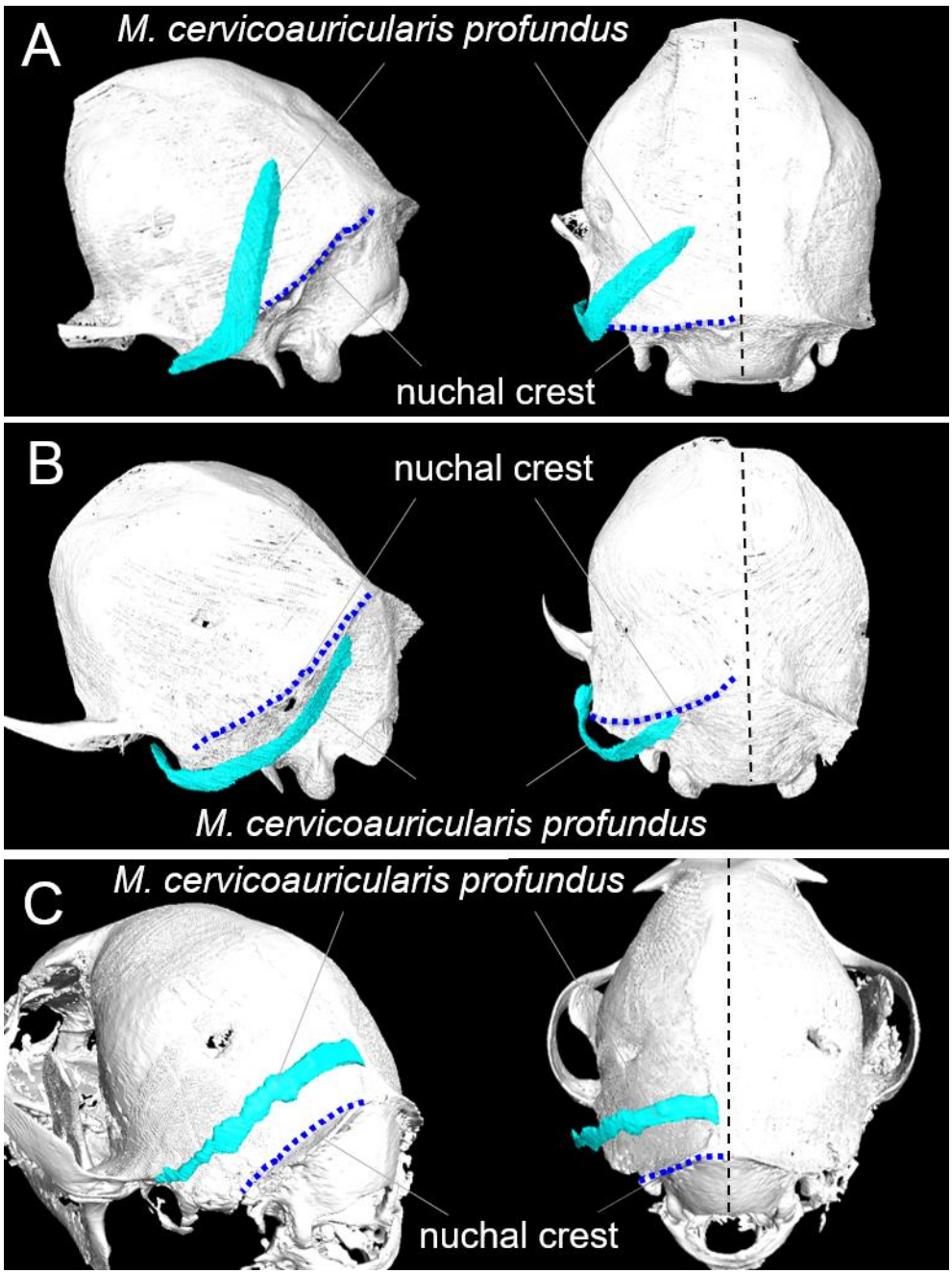

Fig. 8 Comparison of *M. cervicoauricularis profundus* topologies in left lateral (left column) and dorsal (right column) views.

Dotted blue line indicates the nuchal crest, and the dashed black line

indicates the midline. (A) *Eonycteris spelaea*. (B) *Rousettus leschenaultii*. (C) *Cynopterus sphinx*.

2.4 Discussion

The results showed that *E. spelaea*, *R. leschenaultii*and *C. sphinx*, have three components for the cervicoauricularis muscles (Figs. 5, 6 and 7). In the previous report (Chi et al., 2023), I argued that the number of the cervicoauricularis muscles in the common ancestor of laurasiatherians is three. Four muscles are present in rhinolophoid bats (Schneider, 1960; Schneider & Möhres, 1960). However, given that *C. sphinx* and yangochiropteran bats have three muscles, I proposed that the ancestral condition of bats should be three and that the four-muscle condition observed is only a derived condition for bats (Chi et al., 2023). As only one pteropodid species was studied (Chi et al., 2023), further investigation on other pteropodids is necessary to confirm this tentative hypothesis.

The three-muscle condition found in *E. spelaea* and *R. leschenaultii* supports my hypothesis that the three-muscle condition is the ancestral pattern of pteropodids and the whole clade of extant bats. In *E. spelaea* and *C. sphinx*, all the three cervicoauricularis muscles, namely *M. cervicoauricularis superficialis*, *M. cervicoauricularis medius*, and *M. cervicoauricularis profundus* originate from the midline (Figs. 5, 7, 8A and 8C). However, the *M. cervicoauricularis profundus* in *R. leschenaultii* does not originate from the midline but from the nuchal crest (Figs. 6, 8B). I observed that the major difference among the three species was the degree of separation of each cervicoauricularis muscle. In *E. spelaea*, the *M.*

cervicoauricularis superficialis does not cover the *M. cervicoauricularis medius*, and the *M. cervicoauricularis medius* slightly covers the rostral region of the *M. cervicoauricularis profundus* (Fig. 5). In contrast, the three cervicoauricularis muscles are clearly separated in *R. leschenaultii* (Fig. 6). Moreover, the *M. cervicoauricularis profundus* does not originate from the midline but from the nuchal crest in *R. leschenaultii* (Fig. 8B). *Cynopterus sphinx* had a low degree of separation between each *M. cervicoauricularis*. Its *M. cervicoauricularis superficialis* covers most of the muscle bundles of the *M. cervicoauricularis medius* and slightly covers the rostral region of the *M. cervicoauricularis profundus* (Fig. 7). In general, the muscles that are separate and independent allow fine control of movements (George & Robert, 2000). Based on click rates, Boonman et al. (Boonman et al., 2014) evaluated that the ability of click-based echolocation of *Cynopterus* is one of the lowest among pteropodids. The major habitat of *Cynopterus* is in the forest where the environment is not completely dark (Elangovan & Marimuthu, 2001); therefore, they do not need to rely heavily on echolocation. Meanwhile, *Eonycteris* and *Rousettus* are cave dwellers (Gould, 1988; Kulzer, 1958) who need to fly in complete darkness where echolocation abilities may be advantageous. Thus, the separation of the cervicoauricularis muscle may reflect the degree of echolocation ability, thereby facilitating delicate and sophisticated ear movements during echolocation.

The *M. cervicoauricularis profundus* originating from the nuchal

crest (CPNC) is uncommon among non-bat laurasiatherian mammals but can be found in various laryngeal echolocating bats (Schneider, 1960; Schneider & Möhres, 1960). Schneider and his co-worker (Schneider, 1960; Schneider & Möhres, 1960) reported that laryngeal echolocating rhinolophoids have different numbers of cervicoauricularis muscle components compared to non-bat laurasiatherians. The most distinctive features of rhinolophoids are that their cervicoauricularis muscles has four components and their two caudal muscles, namely the *M. cervicoauricularis profundus* major and *profundus minor* originate from the nuchal crest. In the horseshoe bat (*Rhinolophus ferrumequinum*), in addition to the *M. cervicoauricularis profundus major* and *profundus minor*, the *M. cervicoauricularis superficialis minor* also originates from the nuchal crest. Its most rostral cervicoauricularis muscle, the *M. cervicoauricularis superficialis major*, originates from the midline. In the trident bat (*Asellia tridens*), the *M. cervicoauricularis superficialis major* and *superficialis minor* originate from the midline, and the *M. cervicoauricularis profundus major* and *profundus minor* originate from the nuchal crest. The mouse-eared bat (*Myotis myotis*), which is a member of yangochiropterans, only have three muscles (the *M. cervicoauricularis superficialis*, *M. cervicoauricularis medius*, and *M. cervicoauricularis profundus*) as reported by Schneider (Schneider, 1960) (the nomenclature refers to the previous study by Chi et al., 2023). In *M. myotis*, the two rostral muscles, *M. cervicoauricularis superficialis* and *M. cervicoauricularis medius* originate

from the midline, whereas the most caudal *M. cervicoauricularis profundus* originates from the nuchal crest. Therefore, the most caudal or two caudal cervicoauricularis muscles originate from the nuchal crest instead of the skull midline in laryngeal echolocating bats (*R. ferrumequinum*, *A. tridens*, and *M. myotis*).

CPNC should arguably perform differently from the case originating from the midline. In general, the key role of the *M. cervicoauricularis* is to raise the ear pinna (Budras et al., 2013, 2012; Evans & de Lahunta, 2013; Holland & Waters, 2005). In this regard, CPNC helps pull the ear pinna caudally in the horizontal plane. Horizontal ear movements are widely observed in bats during echolocation. *Rousettus* is capable of multiangle ear movements, which possibly allows for the quick localization of the finest sounds (Kulzer, 1958). The big brown bat (*Eptesicus fuscus*), a member of yangochiropterans, increases interpinna separation (caudal movements of the pinnae in horizontal plane) as the echolocated target approaches (Wohlgemuth et al., 2016). These reports suggest that recruiting horizontal movements of the ear can increase the access to sound information and may be related to the ability of active echolocation. Phylogenetically, *Eonycteris* is more closely related to *Rousettus* than to *Cynopterus* (Almeida et al., 2011). However, the *M. cervicoauricularis profundus* originates from the midline of the skull in *E. spelaea* and *C. sphinx* (Fig. 8A, 8C) but originates from the nuchal crest in *R. leschenaultii* (Fig. 8B) and in other laryngeal echolocating bats (*R.*

ferrumequinum, *A. tridens*, and *M. myotis*). As noted earlier, *Rousettus* uses tongue-click echolocation and wing-click echolocation (Boonman et al., 2014; Yovel et al., 2011).Based on click rates, the ability of click-based echolocation of *Rousettus*is arguably higher than that those of *Eonycteris* and *C. sphinx* (Boonman et al., 2014).CPNC, which has been acquired independently in laryngeal echolocating bats and *Rousettus*, may be a key characteristic for sophisticated active echolocation behavior.

In this study, I confirmed that the three-muscle condition of the cervicoauricularis muscles is universal in pteropodids and ancestral for extant bats. So far, the four-muscle condition is unique to rhinolophoids, the sister group for pteropodids, and it is confirmed to be a derived condition for bats. Hence, the homology of the four muscles and their functional significance for echolocation are largely unclear. Further embryological and anatomical studies on the caudal auricular muscles in various rhinolophoids are required to shed light on these issues.

General conclusion

To understand the morphology, homology, and diversity in caudal auricular muscles of bats, microCT study, gross dissection, and fetal anatomy were conducted and the following results were acquired.

The three-muscle pattern of *M. cervicoauricularis* in a greater-nosed fruit bat (*Cynopterus sphinx*) was provided through microCT and gross dissection. By comparing with other non-bat laurasiatherians, these bats retain the ancestral condition for ear muscle attachment pattern. However, the three-muscle pattern was different from the rhinolophoids, which equip the four-muscle pattern.

The homology in caudal auricular muscles of bats were provided through the comparison of fetal anatomy in *C. sphinx* and mouse (*Mus musculus*). Observations on fetal anatomy allowed me to address the homologies of the cervicoauricularis muscles among mammals. the innervation pattern of the three cervicoauricularis muscles in *M. musculus* is comparable to those in *C. sphinx* and *M. myotis* (Schneider & Möhres, 1960). Given the homogeneity of the cervicoauricularis muscle numbers and innervation patterns among the three species, the three muscles' nomenclature in *C. sphinx* should be considered as the *cervicoauricularis superficialis*, *cervicoauricularis medius*, and *cervicoauricularis profundus*, as in *M. musculus*.

The diversity of caudal auricular muscles in bats was provided

through microCT and gross dissection on other two pteropodids, the cave nectar bat (*Eonycteris spelaea*), and Leschenault's rousette (*Rousettus leschenaultii*). By comparing the association between pattern and echolocation behavior, I found that the degree of separation in cervicoauricularis muscles is related to the ability of echolocation, the stronger the ability, the higher the degree of muscle separation. The performance of echolocation decreases from *Rousettus* to *Eonycteris* to *Cynopterus*. Meanwhile, the separation degree of cervicoauricularis muscles decreases from *Rousettus* to *Eonycteris* to *Cynopterus*.

The *M. cervicoauricularis profundus* originating from the nuchal crest (CPNC) is uncommon among non-bat laurasiatherian mammals but can be found in various laryngeal echolocating bats (Schneider, 1960; Schneider & Möhres, 1960). Most caudal or two caudal cervicoauricularis muscles originate from the nuchal crest instead of the skull midline in laryngeal echolocating bats (*R. ferrumequinum*, *A. tridens*, and *M. myotis*) (Schneider, 1960; Schneider & Möhres, 1960). *Rousettus* considered to be the best-ability echolocator among pteropodids also equipped the CPNC. Therefore, the CPNC which has been acquired independently in laryngeal echolocating bats and *Rousettus* may be a key characteristic for sophisticated active echolocation behavior.

This study uncovered the association between the caudal auricular muscles and echolocation in bats and the diversity of muscles found in pteropodids with echolocation ability. To understand the evolution of the

caudal auricular muscles in bats, I performed gross dissection to understand the morphology and diversity of the bats in pteropodids, where a lack of data in the past is, and I obtained innervation information through embryo section reconstruction to understand the homology of the muscles. In the future, it is necessary to conduct research on more species within the yangochiropterans and rhinolophoids to understand the relationships between the diversity of echolocation behavior and the morphology of caudal auricular muscles, to verify hypotheses about the evolution of bat echolocation.

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Abstract in Korean

박쥐는 계통발생학적으로 세 가지 주요 그룹인 pteropodids, rhinolophoids, yangochiropterans로 분류할 수 있다. 다. rhinolophoids와 yangochiropterans는 후두 반향전위 능력이 있으나, pteropodids는 이러한 능력이 부족하다. 섬세한 귀의 움직임은 후두 반향정위에 필수적인 요소이다. 귀의 움직임이 박쥐의 후두 반향정위 능력에 영향을 미친다는 보고가 있으며, 목부위의 근육 특히 목귓바퀴근육은 이러한 귀의 움직임에 중요한 역할을 한다.

이전에는 코뿔소박쥐과와 양견박쥐과를 포함한 세 종의 후두 반향정위를 가진 박쥐의 뒤귓바퀴근에 대한 연구가 진행되었다. 코뿔소박쥐과에서는 뒤귀근육이 4개의 부위로 구성되나, 양견박쥐과에서는 세 가지 근육에 의해서 구성되는 것으로 알려져 있다. 지금까지, 후두 반향정위를 사용하지 않는 날다리박쥐과에 대한 뒤귓바퀴근의 형태학과 신경지배에 관한 연구는 없었다. 따라서 본 연구에서는 날다리박쥐과 동물에서 뒤귓바퀴근의 구조와 이에 대한 신경분포를 확인하고자 한다. 이를 통해 박쥐의 세 가지 주요 그룹의 형태학적 데이터를 비교하여, 귀 근육

형태학적 관점에서 박쥐의 반향정위의 진화를 이해하고자 한다.

제 1장에서는 diffusible iodine-based contrast-enhanced computed tomography (diceCT)를 사용하여 *Cynopterus sphinx*의 목귓바퀴근의 육안 구조를 확인하고, 면역조직화학적으로 염색된 serial fetus sections의 3D 재구성을 통해 목귓바퀴근의 신경분포를 확인하고자 하였다. 후두 반향정위를 가진 박쥐에 대한 연구에서는 코뿔소박쥐과가 네 개의 목귓바퀴근을 가지고 있고, 양견박쥐과가 세 개의 목귓바퀴근을 가지는 것을 확인하였다. 날다리박쥐과인 *C. sphinx*에서 세 개의 목귓바퀴근을 관찰하였으며, 이를 통해 목귓바퀴근을 구성하는 근육 및 신경공급 패턴은 박쥐가 아닌 boreoeutherian 포유류 및 양견박쥐과와 유사함을 알 수 있었다. 이러한 연구 결과는 날다리박쥐과와 양견박쥐과가 일반적인 박쥐가 아닌 boreoutherian 포유류의 형태를 유지하고 있고, 코뿔소박쥐과만이 파생된 조건을 갖고 있다는 것을 시사한다. 최근까지 후두 반향정위를 가진 박쥐의 목귓바퀴근에 대해 특정 명칭을 사용했지만, 코뿔소박쥐과를 제외한 박쥐가 아닌 boreoutherian와 박쥐들 간의 공통성을 감안했을 때 (즉, *M. cervicoauricularis superficialis*, *M. cervicoauricularis medius*, and *M.*

cervicoauricularis profundus) 기존의 명명법을 사용하는 것이 타당하다고 생각된다.

제 2장에서, 박쥐 중에서도 후두 반향정위는 rhinolophoids 뿐만 아니라 yangochiropterans과 pteropodids에서도 나타난다. *Rousettus*는 혀클릭(tongue click) 행동을 통하여, 반향정위를 사용할 수 있는 유일한 pteropodid로 알려져 있었지만 최근 연구에 따르면, 많은 종의 pteropodid는 날개 클릭 행동을 통해 반향정위를 할 수 있다고 보고되고 있다. 반향정위를 연구하는 논문에 따르면, 반향정위에 대한 박쥐의 섬세한 귀의 움직임은 매우 중요하며 이러한 귀의 움직임을 결정하는 중요한 근육이 목귓바퀴근(*M. cervicoauricularis*)이라고 알려져 있다.

본 연구에서는 피물방울박쥐과 (*Cynopterus sphinx*, *Eonycteris spelaea*, *Rousettus leschenaultia)*의 3가지의 종에서 목귓바퀴근의 육안 해부학적 구조를 관찰하여, 후두 반향정위를 보이는 피물방울박쥐들 간에 목귓바퀴근의 구조가 다양하게 나타나는지, 그리고 피물방울박쥐과와 후두 반향정위를 보이는 박쥐들 간에 차이가 있는지 조사하였다. 조사 결과 혀클릭 행동을 보이는 *R. leschenaultii*에서 깊은목귓바퀴근(*M. cervicoauricularis profundus*)이 목덜미능선(nuchal crest)에서

기원하고, 날개클릭 행동을 보이는 *C. sphinx*와 *E. spelaea*에서는 머리뼈의 정중선에서 기원하는 것을 확인하였다. 일반적으로 고주파의 혀클릭 행동이 날개 클릭 행동에 비해 초음파 효율이 뛰어나다고 알려져 있다. 목덜미 능선에서 기원하는 깊은목귓바퀴근은 박쥐가 아닌 laurasiatherian과 같은 다른 동물들에서는 흔하지 않지만, 후두 반향정위를 나타내는 박쥐들에서 확인할 수 있었다. 이러한 이유로 *R. leschenaultii*와 후두 반향정위을 이용하는 박쥐에서 발견된 깊은목귓바퀴근은 박쥐의 정교한 능동적 반향정위 행동을 위한 중요한 특징일 수 있음을 확인하였다. 목귓바퀴근육은 박쥐에서 다양한 형태로 나타나며, 고음파에 의한 정위행동에 연관된다. 원시적인 날개클릭 행동을 보이는 *C. sphinx*는 다른 박쥐가 아닌 lauasiatherian과 유사한 세 개의 근육을 갖는다. 혀클릭 행동을 보이는 박쥐 (*R. leschenaultii*)도 세 개의 근육 구성 요소를 가지며, 특히 깊은목귓바퀴근육을 통해 능동적인 방향정위 나타낸다. Yangochiropterans (*Myotis myotis*) 또한 혀클릭 행동을 보이는 박쥐와 유사한 형태의 3개의 목귓바퀴근육 및 깊은목귓바퀴근육을 가지고 있었다. 가장 복잡한 후두 반향정위을 나타내는 비갈락코량류는 네 개의 목귓바퀴근육을 가지며, 깊은목귓바퀴근육에 의한 능동적 반향정위 조절 능력이 있었다.

따라서, 목귓바퀴근육를 구성하는 근육의 수와 기원의 위치는 박쥐의 공명 기능과 진화적으로 연결될 수 있음을 시사한다.

이 연구는 기존의 명명법을 유지하면서 비갈락코량류의 목귓바퀴근육의 다양성을 제안하였다. 이 결과는 앞으로의 박쥐의 귀 근육 연구에 대한 기초를 마련하며, 다양한 종류의 박쥐에서의 귀 근육 연구가 이 근육의 기능, 상동성 및 진화에 대한 더 깊은 이해를 제공할 것으로 생각된다.

주요어: 비교해부학, 반향정위, 진화, 머리뼈

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