# DYNAMIC BEAM FORMING IN FREE-TAILED BATS

A Thesis

by

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# ABSTRACT

There is behavioral evidence that echolocating bats can manipulate the acoustic projection pattern of their sonar pulse emissions, but the mechanism(s) for this are unknown. I hypothesized that the Mexican free-tailed bat (*Tadarida brasiliensis*) achieves this by finely adjusting the shape of its mouth (beam-forming) in a behavior akin to supralaryngeal speech motor control by humans. This hypothesis arose from my discovery that *Tadarida brasiliensis* raise their noses and lips preceding each echolocation pulse and that they possess a hypertrophied set of specialized facial muscles possibly analogous to the *levator labii aleque nasi*. I investigated whether this muscle complex 1) is active during sonar performance, 2) displays anatomical and histological specializations consistent with the high-speed demands of echolocation, and 3) can effectively perform beam-forming through fine manipulations of the nose and mouth. Firstly, EMG recordings from awake echolocating bats confirmed that these muscles were activated in a temporally precise coordination with pulse emissions. Secondly, I described the anatomical organization of the muscle complex, its origin and insertions, and its innervation patterns. Histochemical analyses confirmed that these were fast-twitch muscles, as expected for muscles adapted for rapid contractions for extended periods. Lastly, I directly measured how changes in face shape affected the sonar beam-width. This muscle complex allows bats to lift the nose tip to create a small aperture, producing a wide-angle beam, or to lift both the nose and the upper lips simultaneously creating a wider aperture but narrower beam. I confirmed that for a

typical pulse (downward FM sweep, 50-20 kHz), raising and pulling back the lips narrowed the projection beam relative to just raising the nose tip with lips held down. These results confirm that *Tadarida* possesses a specialized supralaryngeal neuromuscular apparatus for sonar beam-forming.

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# INTRODUCTION

A major question in neuroscience is how the brain encodes and coordinates complex motor patterns. Mammalian vocal motor programs are a unique example of neural coordination of multiple muscle groups based on feedback from several sensory systems to generate complex sounds. Vocal motor patterning and flexibility is of especially great importance to humans, and the ability to produce and use speech is one of the most robust distinctions between humans and the rest of the animal kingdom. All terrestrial mammals use largely the same sets of respiratory and laryngeal muscles, and most also incorporate the movement of supralaryngeal components, such as the tongue, jaw, lips, or nose to shape the acoustic properties of the outgoing sound (Smotherman, 2007). However, despite the large number of vocal mammals, very few produce vocalizations with flexibility and control on par with human speech. Echolocating bats, such as the Mexican free-tailed bat (*Tadarida brasiliensis*) used in this study, offer a unique opportunity to study neural control of the voice because they must constantly adjust echolocation pulse acoustics and timing to gain an accurate acoustic picture of their surroundings or of insect prey while hunting. By exploring the neurophysiological mechanisms and details of bat vocalizations we thus gain important comparative insights into such complex mammalian vocalizations as human speech.

Acoustic properties of bat echolocation calls vary phylogenetically, with groups and species of bats producing widely different call types, and also individually a single bat can alter the properties of its echolocation pulses based on variations in the environment

or behavioral circumstances. The acoustic structure of the outgoing pulse greatly influences sonar range and resolution, and therefore it directly impacts a bats navigational performance in different habitats and across diverse behavioral contexts. Most bats can manipulate their pulse acoustics in several subtle ways, such as changes to parameters such as call frequency bandwidth, intensity, duration and repetition rate (Griffin, 1958; Simmons et al., 1979). The behavioral and ecological significance of these vocal behaviors have been extensively documented in a wide variety of bat species, but there is one additional parameter that is of equal importance but has received far less attention, namely the directionality of the outgoing sonar pulse. Studies of directionality are regrettably few, but this important additional level of vocal control over the acoustic field of view, or the echolocation beam pattern, allows bats to finely tune the direction and scope of an echolocation beam. They accomplish this in two ways: firstly beam forming can be accomplished by simply adjusting the peak call frequency and raising the bandwidth of the outgoing pulse (Mogensen and Møhl, 1979), a mechanism for focusing the beam based on the fact that higher frequencies show greater off-axis attenuation and therefore tend to produce relatively louder echoes from directly in front of the bat. Alternatively, by lowering the pulse frequency bats can increase the loudness of off-axis echoes and thereby effectively broaden their sonar beam. This behavior has been seen mostly in vespertilionid bats (Jakobsen and Surlykke, 2010; Motoi et al., 2017), but also has drawbacks such as shifting the frequency of the returning echoes away from the most sensitive region of the bats cochlea. An alternative mechanism for beam forming can be achieved by manipulating

fine features of the face to adjust the size and shape of the emitter (nose or mouth). Through manipulation of the emitter shape bats can adjust the dimensions of the outgoing sonar beam – small diameter emitters produce a larger, less focused beam, while increasing the diameter of the emitter focuses and decreases the angle of the beam (Strother and Mogus, 1970). These changes in mouth shape do not need to be perfectly symmetrical to produce an effective change in beamshape, and indeed so far only changes in the vertical axis have been documented. For example, the frequency-modulated (FM) echolocating Bodenheimer's pipistrelle bats (*Hypsugo bodenheimeri*) increase the gape of the mouth by lowering the lower jaw when flying into a confined space which thereby narrows the beamwidth in the vertical plane, and they decrease mouth gape when exiting the cluttered environment to broaden their viewfield in open spaces (Kounitsky et al., 2015). However there are doubts about the general efficacy of using mouth gape angle for making anything more than crude adjustments in beamwidth (Kloepper et al., 2014). Still, gape angle remains the only supralaryngeal mechanism by which a bat has been shown to be able to alter an echolocation beam. Incorporating fine manipulations of other face muscles, as in human speech, offers far more possibilities than what can be achieved with gape angle alone, but this hasn't been investigated.

Most of what is known about active beam forming by movements of the face comes from studies of nose-emitting bats. Several families of bats, such as the Phyllostomidae, Rhinolophidae, Hipposideridae, and Megadermatidae, emit echolocation pulses through the nose, which is a big advantage when a bat has to fly while holding food in its mouth. Many of these bats have specialized structures called

nose-leaves surrounding the nostrils, which serve as sophisticated baffles to alter the shape of the outgoing echolocation beam. Japanese greater horseshoe bats (*Rhinolophus ferrumequinum nippon*) emit a constant frequency- frequency modulated (CF-FM) pulse and have been shown to increase the width of the echolocation beam during the terminal phase of prey capture without adjusting frequency (Matsuta et al., 2013). Apparently they accomplish this beam pattern change by slightly changing the nose leaf structure. Greater horseshoe bats (*Rhinolophus ferrumequinum*) were also shown to rotate the lancet of the nose leaf *in vivo* to achieve a significant change in beam width (Gupta et al., 2013), and to move the anterior leaf of their horseshoe shaped nose leaf such that the aperture size of the nose leaf changes significantly – which is theoretically likely to affect the beam pattern of outgoing echolocation pulses (Feng et al., 2012). The Pale spear-nosed bat (*Phyllostomus discolor*) can focus its echolocation beam without altering the spectral components of the echolocation call, and it is able to move various parts of the nose leaf volitionally. It was hypothesized that the bat moves the nose leaf to manipulate the projection pattern of the echolocation pulse as the bat approaches its prey (Linnenschmidt and Wiegrebe, 2016). Other phyllostomids, including the trawling long-legged bat (*Macrophyllum macrophyllum*) and the fringe-lipped bat (*Trachops cirrhosis*), bend their nose leaves vertically towards prey, along with the head and ears, providing additional evidence that the nose leaf is recruited to focus the sound beam towards prey (Surlykke et al., 2013; Weinbeer and Kalko, 2007). Still, movement of the nose leaf is a limited mechanism for alteration of the beam pattern as nose leaf movements only accommodate small changes in the echolocation beam and the pulse

acoustics of many leaf nosed bats are spectrally more simple than those produced by mouth emitters (Metzner and Schuller, 2010). There remains much to be discovered about how the nose leaf is involved in shaping an echolocation beam, and the field still lacks conclusive evidence that bats intentionally move the nose leaf to manipulate beam shape, but these studies present strong evidence to support this hypothesis. Additionally, the current literature highlights the importance of beam forming to bats, but fails to provide details of how and when mouth-emitting bats might utilize manipulation of the beam pattern.

The general mammalian vocal motor pathway, such as that of a rodent or a cat, begins with limbic activation that starts at the anterior cingulate cortex and acts through midbrain central pattern generators to coordinate the muscles that produce a specific vocalization (Newman, 2010) (Figure 1, blue pathway, (Schwartz and Smotherman, 2011) and Figure 2, (Jurgens, 2009)). Rodents, which have been extensively studied, have a standard mammal system that does not involve sensorimotor control of vocalizations, and like the majority of vocal mammals do not make use of the supralaryngeal system, or elements of the head and face, but rather produce "pre-programmed" vocalizations (Gonzalez-Lima, 2010). Mammalian use of supralaryngeal facial movements, other than simple changes in gape height, to adjust spectral components of vocalizations is limited to primates (Jurgens, 2009). While primates retain the basic limbic-driven vocal motor pattern generator, they also make special use of supralaryngeal musculature to manipulate vocalizations, which greatly increases the complexity of both the vocal repertoire and the neural pathway involved (Simonyan et

al., 2012). Primates in general use a wide variety of facial movements as part of their communication behaviors, and to varying degrees these facial movements have become coupled to vocalization patterns. Humans and a few other primates make use of the motor cortex to drive the supralaryngeal musculature, such as the lips and tongue, to form highly complex, flexible vocalizations, such as speech (Figure 1, green pathway, (Schwartz and Smotherman, 2011). It is hypothesized that the coupling of facial expressions with vocalizing was initially achieved via parallel activation of the limbic vocal motor pathways and corticospinal activation of face muscles. At some point, the evolution of human speech appears to have required an almost complete switch from the limbic system to the neocortical vocal motor pathway, which was probably necessary to accommodate vocal learning. Humans still use the limbic vocal pathway to produce non-speech vocalizations such as laughter, crying or grunting, but much remains unknown about the human and mammalian vocal motor pathways in any animal. From a comparative standpoint, studies of supralaryngeal vocal control are essential to our understanding of the mechanisms and neural contributions underlying the evolution and production of speech sounds. So far the extent of detailed physiological studies in this field of research has been limited by the unfeasibility and restrictions of using human and primate subjects for invasive and thorough neurophysiological experimentation. Here, I propose that echolocating bats, like the free-tailed bat *Tadarida brasiliensis*, provide both the supralargyneal vocal control and aptitude for use within a laboratory setting to make a significant contribution to furthering our understanding of the motor cortex's involvement in the production of complex vocalizations like human speech.

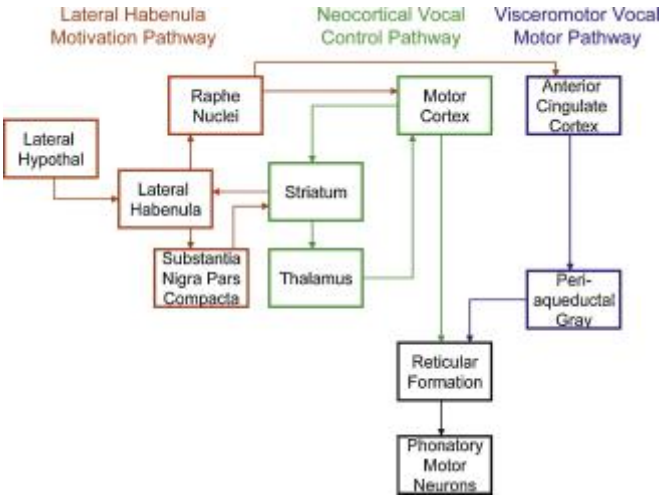


**Figure 1**. The mammalian vocal motor pathway from (Schwartz and Smotherman, 2011). The standard mammalian vocal pathway is shown in blue, with hypothesized contributions of a neocortical pathway, such as that found in primates and bats, shown in green and the motivation pathway shown in red.

Call production at the laryngeal level in bats follows the mammalian pattern described above, with some distinct modifications that make it possible for them to produce and tightly control spectral features and timing of ballistic, ultrasonic echolocation pulses. For example, anatomical connections between motor subsystems like those involved in respiration, wing stroke cycles, and echolocation call emission is much more pronounced in bats, as their echolocation behavior is usually coupled with other complex motor programs like active flight and phase-locked to the respiratory cycle, unlike in the majority of terrestrial, vocal mammals (Smotherman et al., 2006). Additionally, most of the brainstem areas involved in echolocation pulse production are also innervated by components of the auditory system which demonstrates the important role of auditory feedback for shaping pulse acoustics, including directionality (Metzner

and Schuller, 2010). I show here that some echolocating bats, like primates, may also have a supralaryngeal component to echolocation pulse production by using a complex of facial muscles, hypertrophied compared to other mammals of comparable size, to control the directionality, or beam pattern, of echolocation pulses. I demonstrate here that the neural control of this muscle complex begins in the motor cortex and likely follows the same neocortical motor pathway involved in primate production of flexible, highly complex sounds including human speech (Figure 1, red pathway, (Schwartz and Smotherman, 2011)). This recruitment of muscles of the face to form echolocation pulse beams may be a unique example of convergent evolution of the vocal neural architecture for different ecological pressures.

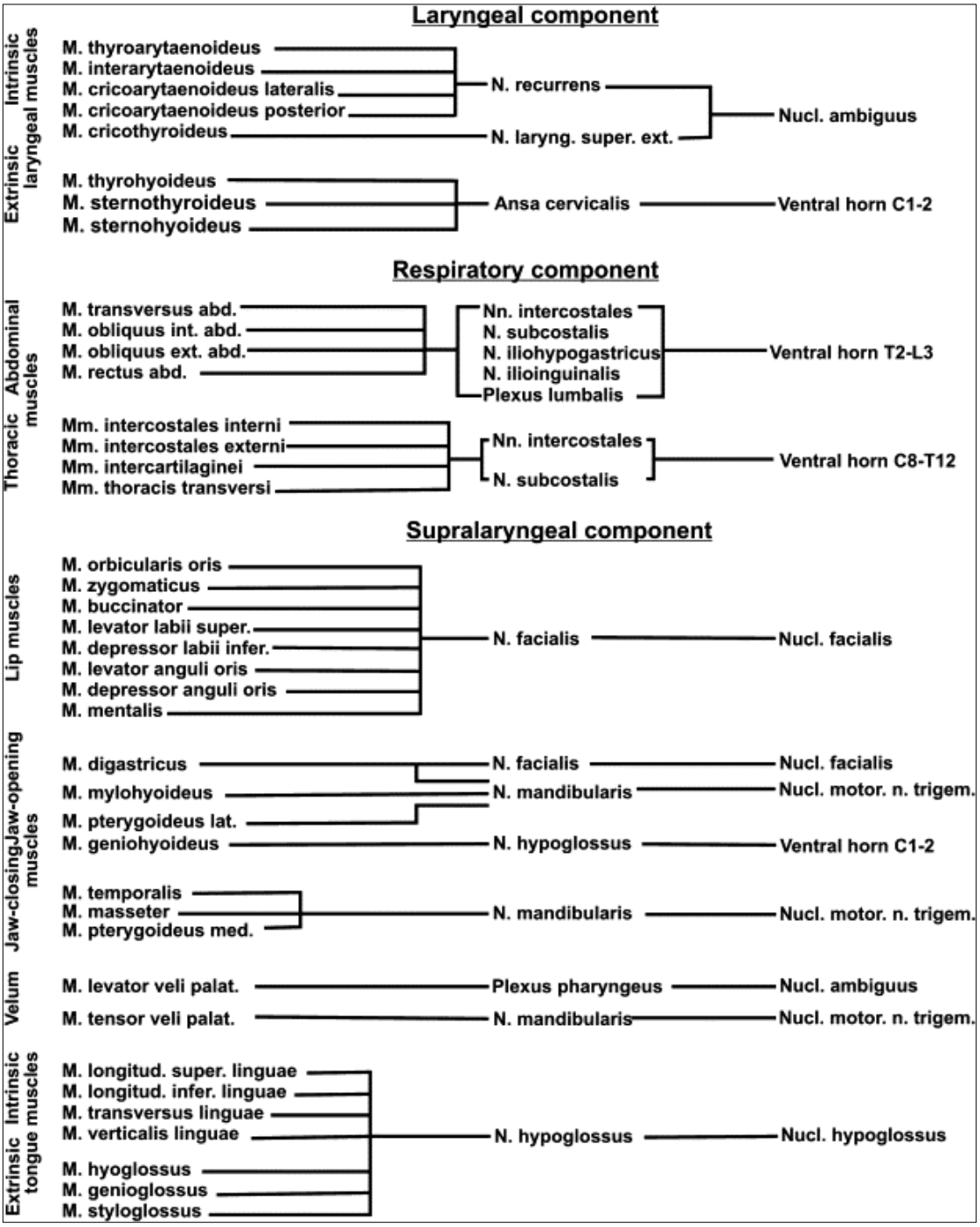


**Figure 2.** Levels of vocal control from (Jurgens, 2009)**.** Vocal control can be organized into three levels: the lowest being the respiratory component, then the laryngeal component, and finally the supralaryngeal component. Each of these levels is comprised of a group of muscles which are innervated and controlled by distinct areas of the brain, listed here.

In this study I focus on a muscle complex I believe to be the *levator labii superioris alaque nasii* muscle of the free-tailed bat, which is likely homologous to the *levator labii superioris* listed in Figure 2, and I describe its unique role in supralaryngeal vocal control of the echolocation beam pattern. I used high-speed video to document that free-tailed bats lift the tip of the nose and raise the lips with each echolocation pulse, rather than simply lowering the jaw to emit sound. I used electromyography to confirm that the *levator labii superioris alaque nasii* is active during each echolocation pulse, and I used immunohistochemistry to determine whether this muscle was biologically adapted to support the energy demands of echolocation, which requires high-speed, highly aerobic muscle performance. I describe in detail the morphology and innervation patterns of this muscle and its subdivisions, showing how it is arranged to manipulate the shape of the face. I then show that this manipulation of the face, i.e. raising the nose and upper lips (Figures 3 and 4), has an effect on the projection pattern of an outgoing sound, and is very likely used by free-tailed bats to finely tune the size and shape of the echolocation beam pattern. Lastly, I show preliminary evidence that control of this

muscle complex lies in the motor cortex along with other mobile components of the face such as the ears and lower jaw.

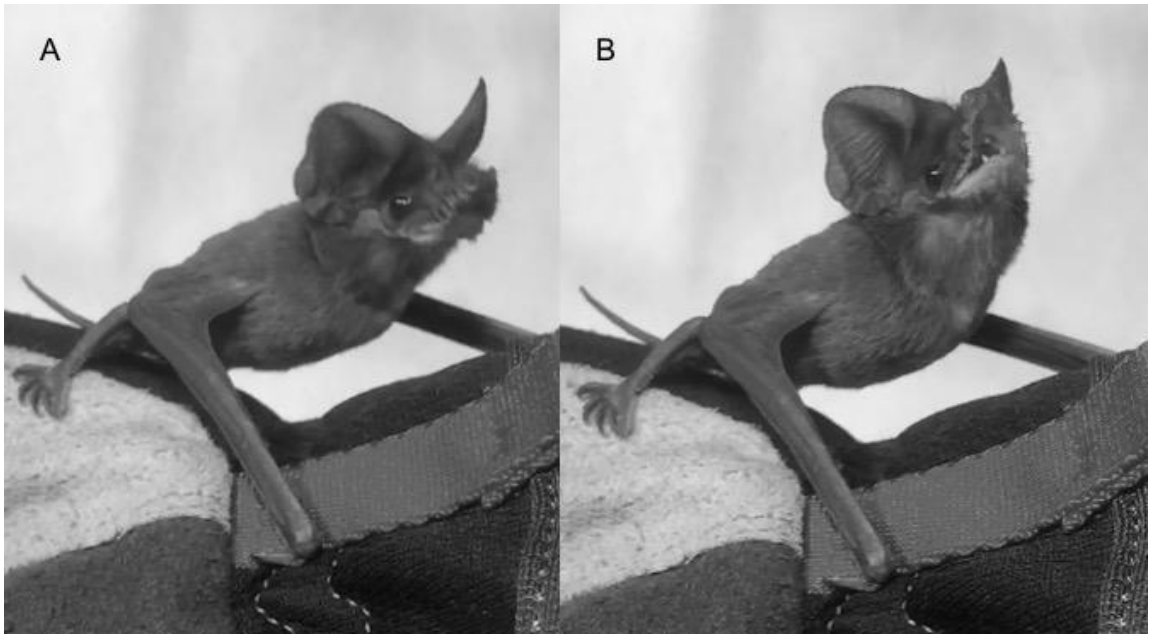


**Figure 3.** *Tadarida* faces during echolocation pulse emission while stationary. (A) Silent bat with nose and lips down. (B) Bat producing an echolocation pulse with nose lifted and lips slightly raised.

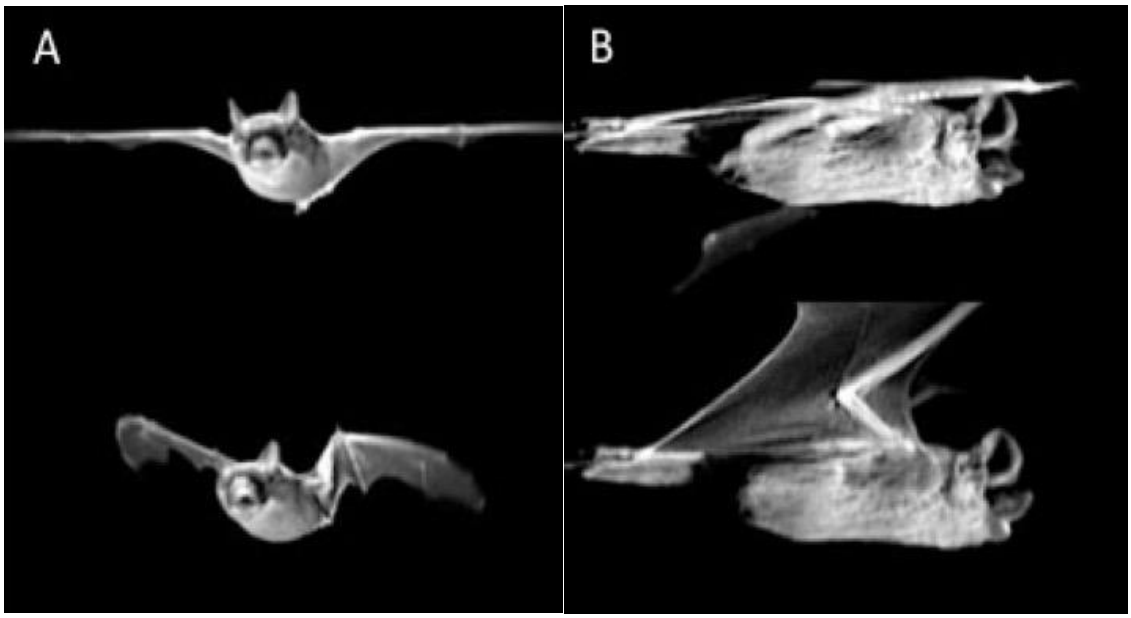


**Figure 4.** *Tadarida* echolocating in flight. (A) Frontal aspect and (B) lateral aspect of body position while vocalizing in flight. Bottom panels show rostrum lifted during an echolocation pulse.

# MATERIALS AND METHODS

## Animal Husbandry

For behavioral and neurophysiology experiments I used a captive colony of free-tailed bats (*Tadarida brasiliensis*) housed in the Biology department vivarium on the College Station campus of Texas A&M University. The colony consisted of approximately 30 individuals, both male and female. The bats occupied two rooms (4x5x3 $m^3$) with regulated light-dark cycles to mimic the natural external photoperiod and are temperature and humidity controlled. Bats were free to fly within the room, and artificial roost sites are available. Bats were trained to feed themselves, and received a diet of mealworms supplemented with vitamins and essential fatty acids, but did not hunt on the wing in the lab. These bats were collected locally, from the large colony under the Waugh bridge in Houston, Texas, and housed for up to two years. *Tadarida brasiliensis* bats rely on echolocation pulses to produce an auditory map of their surroundings, use the information to capture insect prey and navigate their environments, and readily produce echolocation pulses in the lab. All experiments were carried out according to the National Research Council guidelines (National Research Council, 2011) and were approved by the TAMU Institutional Animal Care and Use Committee (AUP# 2014-146).

## High-Speed Video

High-speed video was captured using an iPhone camera and the Apple iOs slow-motion video application. I also relied on two Basler acA640-120um USB 3.0 cameras with Sony ICX618 CCD sensors mounted inside the flight room. Video was captured at >110 frames per second. For videos of bats in flight, a camera was mounted on a tripod and placed directly in front of a landing platform in a flight room. The flight chamber was a 6x3x1.5$m^3$ room, with walls and ceiling lined with 3-inch acoustic foam. Bats were trained previously to fly across the room twice when released by a handler and land on the platform. During recording bats were released just above the camera, flew across the room and back directly towards the camera, then landed just behind it on the landing platform. For videos of stationary bats, bats were allowed to sit quietly on my gloved hand on a cloth-covered recording platform inside the flight room, under a spot light for optimal lighting. Animals' spontaneous echolocations were recorded during these sessions, and each session lasted 20 seconds. Four trained bats (two males and two females) were used for both in-flight and stationary recordings. Each animal was recorded for five repetitions of each type of recording. From these recordings facial movements, specifically movements of the lips, nose, and jaw, were observed, and the stereotypical movements of the face used to produce echolocation pulses were documented.

## Electromyography and Inactivation

Electromyogram (EMG) activity from the muscles of the nose, the *levator labii superioris aleque nasii* was recorded along with simultaneous ultrasonic recordings while animals actively vocalized in order to determine if the action of this muscle group corresponds to production of or changes in echolocation pulses. A soft silver-wire electrode was placed above these muscles through the skin above the rostrum using a 27-gauge hypodermic needle. Once inserted, the wire was bonded to the skin with veterinary adhesive (to reduce electrical noise from body movements) and the animal was placed in a small cage within an anechoic chamber. The EMG electrode was connected to an amplifier so that the animal was tethered but free to move about the cage. Experimental trials lasted for 30 minutes per bat for each of three bats, during which echolocation pulses and nose muscle EMGs were recorded simultaneously. Spontaneous ultrasonic vocalizations were recorded simultaneously. Vocalizations were recorded using a condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) positioned 10 cm from edge of the cage and oriented toward the center. The bats' vocalizations were digitized and analyzed using the hardware and software package Datapac 2K2 (RUN Technologies, Mission Viejo, CA). Pulses were automatically discriminated from background by applying a fixed threshold to the waveform envelope.

In order to further determine the contribution of this muscle group to the echolocation waveform, the *levator labii superioris aleque nasii* muscle was reversibly inactivated and echolocation pulses recorded. A small amount of a local anesthetic

(Bupivicaine HCl 0.5%, Hospira, Inc., 0.1mL per bat) was injected sub-durally above this muscle to induce temporary paralysis. The drug was allowed to spread and completely inactivate this muscle (3- 5 minutes) before the bat was placed in a small cage within an anechoic chamber and allowed to move and vocalize freely. Again, vocalizations were recorded using a condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) positioned 10 cm from edge of the cage and oriented toward the center. The bats' vocalizations were digitized and analyzed using the hardware and software package Datapac 2K2 (RUN Technologies, Mission Viejo, CA). Pulses were automatically discriminated from background by applying a fixed threshold to the waveform envelope.

**Anatomy (Drawing and Nerve Stain)**

For studies of gross anatomy of nose muscles I used only bats that had been previously euthanized and stored in a freezer. Four animals, two females and two males, were decapitated and the heads kept on ice while being prepared to be stained and photographed. Heads were left on ice for no more than twenty minutes before staining and imaging. When ready to be imaged, skin was carefully removed from the top of the head, between both ears and rostro-caudally from the nose to the cranial ridge. I observed and photographed origins and insertions of the muscle complex, surrounding connective tissue and innervation and vascularization patterns using an Olympus SZ61 microscope, and used this information to produce a detailed anatomical drawing of the

muscle complex. Nomenclature of this musculature was based on its location and insertion points and follows Burrows *et al.* (Burrows et al., 2006), though this muscle appears to be hypertrophied in *Tadarida* relative to head size compared to primates and other vocal mammals (Bruintjes et al., 1996; Diogo et al., 2012; Letourneau and Daniel, 1988).

## Histology

### *Hematoxylin and eosin staining (H&E staining)*

A hematoxylin and eosin stain was used to examine muscle fiber organization and diameter (Brueckner). Muscles were excised from four bats (3 females and 1 male) and flash- frozen in isopentane on dry ice before cryosectioning, both in cross-section and longitudinal sections, into 14 μm slices and mounted on Histobond glass slides. Slides were allowed to dry at room temperature for one hour prior to staining. Sections on slides were stained with Mayer's Hematoxylin Solution for 2-5 minutes, then rinsed in warm running tap water for 15 minutes. Slides were then placed in distilled water for 30 seconds and 95% ethanol solution for 30 seconds. Slides were then counterstained with Eosin Y Solution for 2-5 minutes. Slides were then dehydrated and cleared by submersion twice in 95% ethanol for 2 minutes, twice in 100% ethanol for 2 minutes, and twice in xylene for two minutes. Slides were then mounted and cover-slipped with Permount mounting medium and allowed to dry before imaging. Slides were imaged using an Olympus CX41 microscope at 100X magnification, and pictures were taken

using an Infinity 2 microscope camera connected to a computer running Infinity Capture application software (version 3.7.5, Lumenera Corporation). Measurements of fiber size and density were made using NIH Image J (Abràmoff et al., 2004).

### *Antibody staining*

An antibody staining protocol adapted from Behan *et al.* (Behan et al., 2002) was used to determine the relative composition of myosin subtypes in these muscles (Armstrong and Phelps, 1984), which correlates with twitch speed and aerobic capacity. Muscle tissue was excised and flash-frozen in isopentane on dry ice before cryosectioning into 14 μm slices and mounting on Histobond glass slides. Slides were allowed to dry at room temperature for 30 minutes prior to staining. Slides were briefly fixed in pre-cooled acetone for 10 minutes. Acetone was allowed then to evaporate for 20 minutes at room temperature. Sections were incubated with powdered milk in Tris buffered saline (TBS, pH 7.6) for 10 minutes. Excess serum was then drained and the slides were incubated in monoclonal antibody to slow myosin (primary, Sigma Aldrich, MAV1628- Anti-Myosin Antibody, slow muscle, clone NOQ7.5.4D) diluted to 1/2000 in TBS for 30 minutes. Slides were washed three times for five minutes in phosphate buffered saline (PBS). Slides were then incubated in a peroxidase conjugated antibody (secondary, Sigma Aldrich, AP160P- Rabbit anti-Mouse IgG Antibody, HRP conjugate) diluted to 1/50 in TBS for 60 minutes. Slides were again washed three times for five minutes in phosphate buffered saline (PBS). Vector SG Peroxidase substrate solution (from the Vector SG Peroxidase (HRP) Substrate Kit, SK-4700) was applied and the

reaction progress was monitored visually at room temperature by microscopic examination over 2-15 minutes. Sections were washed in running tap water for 1 minute to stop the reaction and were then dehydrated using a series of graded alcohols for two minutes each: 70% ethanol, 95% ethanol, and 100% ethanol. Finally, slides were cleared in xylene for 5 minutes, mounted and cover-slipped with Permount mounting medium. Slides were imaged using an Olympus CX41 microscope at 100X magnification, and pictures were taken using an Infinity 2 microscope camera connected to a computer running Infinity Capture application software (version 3.7.5, Lumenera Corporation). This protocol turns slow oxidative/Type I fibers black, numbers of which were compared to positive and negative controls to determine the fiber type of the *levator labii superioris aleque nasii* muscle complex.

## Beam Width Measurements

Beam projection patterns are measures of root mean square (RMS) sound pressure levels at points around a sound emitter, in this case the bat mouth. Since live bats do not produce echolocation pulses when head-fixed in the lab, and since I needed to keep the distance from the animal nose to the microphone precise and consistent in order to collect accurate sound level data, I chose not to use live bats for this section. Here, beam patterns were measured using three ethanol fixed free-tailed bat heads, collected from animals that had been euthanized previously and stored in a freezer. Animal tongues and larynxes were removed to eliminate their effect on beam projection

patterns as they can create additional baffles, possibly changing the pattern of the outgoing sound. Animal noses were lifted and held in place with a small stich to the back of the head and the lips left lying in a natural position against the teeth with the jaw open ("nose up" condition) to mimic the position of the nose when the *levator labii superioris aleque nasii* is partially contracted, or the nose and the lips were lifted and held in place with three small stiches ("lips up" condition). Heads were mounted on the arm of a custom built automated arm controlled by a microcontroller (Arduino UNO) programmed to move the head with a stepper motor in 10-degree steps around a 180-degree arc, both horizontally and vertically, for a total of 324 different positions. The device was positioned such that the tip of the animal's nose was located 10cm from a microphone to measure how the shape of the mouth influenced projection patterns. Pure tone sounds on constant intensity (25kHz, 30kHz, 35kHz, 40kHz, 45kHz) were played from a small Tweeter speaker attached to the back of the buccal cavity via a 1.5cm polyethylene tube. Sound output levels at the different positions in space were and recorded using a condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) and digitized with a multifunction analog-to-digital converter (X Series, National Instruments, Austin, TX) with recording parameters set by the multichannel recording software Avisoft-RECORDER to 192 kHz sampling rate, 16 bit resolution, while the animal head moved across the steps of the moving arm. Intensity of the sound at the microphone was measured in as the RMS voltage signal (in microamps (μA)). 30 (10 for each bat head) stimulus presentations at each position were averaged and then

mapped onto a radial grid to show the projection pattern of the outgoing beam of sound in the vertical and horizontal directions for each of the two facial configurations.

## Cortical Stimulation

Six bats, three females and three males, were used to investigate whether or not the nose muscle could be activated by direct stimulation of the face region of the motor cortex. To do this, I followed a modified version of the intracortical microstimulation (ICMS) technique used by (Tennant et al., 2011). First, animals were pretreated with an intraperitoneal injection of atropine (.05 mg/kg) before being anesthetized with vaporized isoflurane. Once asleep the animals were placed in a custom-built stereotaxic apparatus. Lidocaine (2 mg/kg) with epinephrine was injected under the scalp and a surgery was performed to reflect back the skin and muscle tissue above the target area and a 1.5 mm craniotomy was drilled into the skull above the putative area of the motor cortex. The craniotomy was filled with a warm (37° C) silicone oil to prevent desiccation. A headbolt was attached to the skull caudally above the cerebellum with dental cement 1 mm behind the craniotomy to stabilize the skull during stimulation and suspend the head in a comfortable position that allowed facial movements associated with vocalizing. ICMS was performed using a bipolar stimulating electrode positioned by a stereotaxic micromanipulator at a series of points distributed linearly along the cingulate sulcus, and at each insertion point the tip was lowered to an initial depth of 200 microns below the cortical surface and then progressively lowered in steps of 50 microns

to a maximum depth of 700 μm. At each site, a 40-ms train of 10 200-μs monophasic cathodal pulses were delivered from an electrically-isolated constant current stimulator at a rate of 1 Hz. Stimulation amplitude was increased from 10 μA up to a maximum of 60 μA or until movements of any muscle were detected. The stimulation amplitude limit was set to 60 μA as only sites where stimulation amplitudes of <50 μA are sufficient to elicit a motor response are considered to be positive in rats, where the ICMS technique is most commonly used (Gioanni and Lamarche, 1985). I started at the rostral-most edge of the craniotomy and after each penetration the electrode was raised out of the brain and moved caudally in 100-micron steps. If no movement was detected at 60 μA the site was considered unresponsive. Each penetration included 10 stimulation sites across 500 microns in depth and took approximately 10 minutes to complete. Each of the six bats received 5-10 rostrocaudal penetrations separated mediolaterally by 100 microns each for a total experimental duration of approximately 50-100 minutes. Throughout the procedure animals were kept anesthetized, heart rate and breathing rate were monitored, and surgical plane of anesthesia was maintained as needed by adjustment of the concentration of vaporized isoflurane. At the end of the experiments the animals were euthanized and their brains processed for histochemical verification of electrode positions.

# RESULTS

## Electromyography and Inactivation Experiment

To understand how the time-course of *levator labii superioris alaque nasii* muscle contractions compares to echolocation pulse production, I used electromyography of this muscle simultaneously with ultrasonic recordings. I recorded 1,306 echolocation pulses from three bats and found that for each single echolocation pulse there was a corresponding contraction of the *levator labii superioris alaque nasii* (Figure 5a). When echolocation pulses were produced in pairs, called "doublets," the muscle contracted only once (Figure 5b). Free-tailed bats typically emit one to four pulses in a single breath, but I didn't see triplets or quadruplets during my recordings likely because animals typically only use them while flying. Muscle action began on average 22 ms before, and overlapped slightly with the recorded sound (SEM < 0.001, Figure 5c). Muscle contractions did not vary with changing echolocation pulse duration, and muscle contractions lasted on average 16ms ($R^2 < 0.001$, SEM <0.001, Figure 5d). Inactivation of this muscle group did not affect number or duration of echolocation pulses produced. We analyzed pulse acoustics and there were no major changes in the major acoustic parameters: duration and timing of pulses, frequency bandwidth, and intensity, which supports the idea that free-tailed bats' use of the lips and mouth during echolocation is related to directionality of the beam.

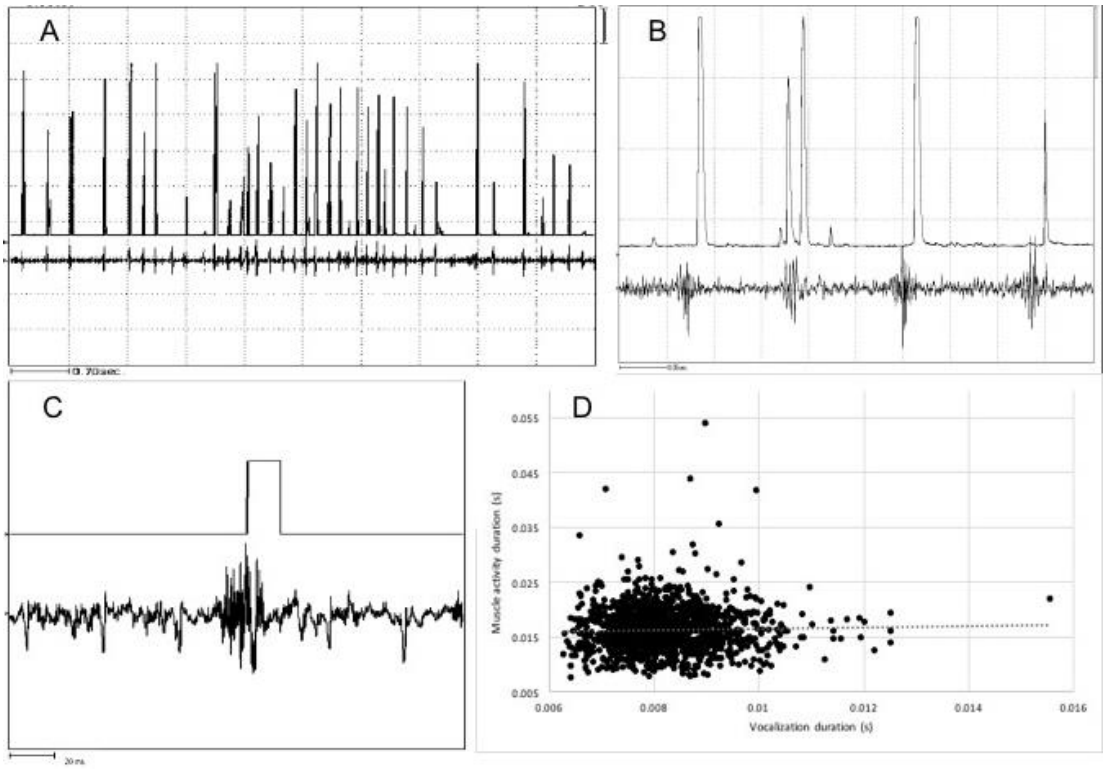


**Figure 5.** Electromyography of the *levator labii superioris alaque nasii* with vocalizations. (A), (B), and (C) Representative recordings of muscle activity (top trace) and simultaneous vocalizations (waveform envelope, bottom trace). (D) muscle activity duration versus vocalization duration, $R^2$ <0.001. N=1,306 echolocation pulses, average onset-to-onset latency= 22ms, SEM <0.001.

## Anatomy

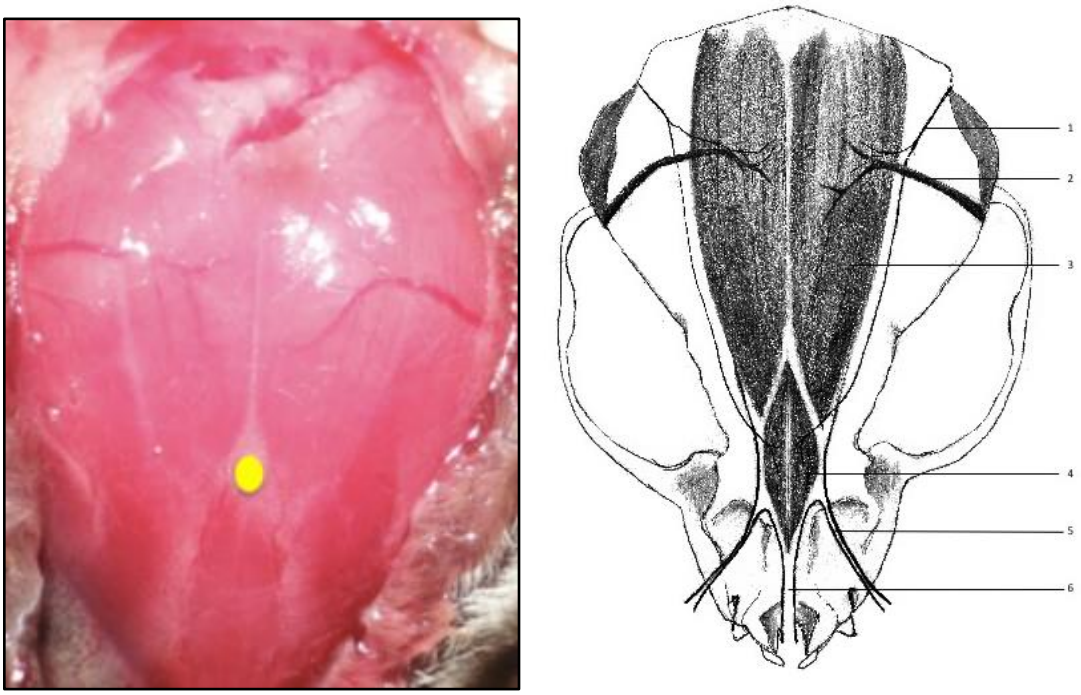


**Figure 6.** The *levator labii superioris alaque nasii* muscle complex. Left: Photograph of the muscle complex, yellow circle marks the division between anterior and posterior portions. Right: Illustration of the muscle complex over the skull, 1. Branch of the facial nerve VII. 2. Branch of the superficial temporal artery. 3. *M. levator labii superioris alaque nasii*, posterior portion. 4. *M. levator labii superioris alaque nasii*, anterior portion. 5. Labial tendon. 6. Rostral tendon.

The *levator labii superioris alaque nasii* muscle in the free-tailed bat is organized into a rostral and a caudal section, each divided into left and right halves (Figure 6). The midline of the muscle complex lies directly superior to the sagittal suture of the skull. The left and right halves of the muscle complex each have their own blood supply from left and right branches of the superficial temporal arteries. The anterior and posterior muscle pairs are innervated by two small branches of the facial

nerve. The posterior portion of this muscle originates from the lambdoidal ridge, and inserts onto the anterior portion medially and onto the upper lips laterally. The anterior portion originates from the anterior edge of posterior portion and inserts onto a cartilaginous pad in the tip of the nose, or the rostral cartilage. Contraction of the entire muscle complex pulls back the nose and lips and produces what I will refer to here as the "lips up" facial configuration (illustration in Figure 7). Contraction of only the rostral portion pulls back the nose while leaving the lips in place against the teeth, and produces the "nose up" configuration (Figure 7).

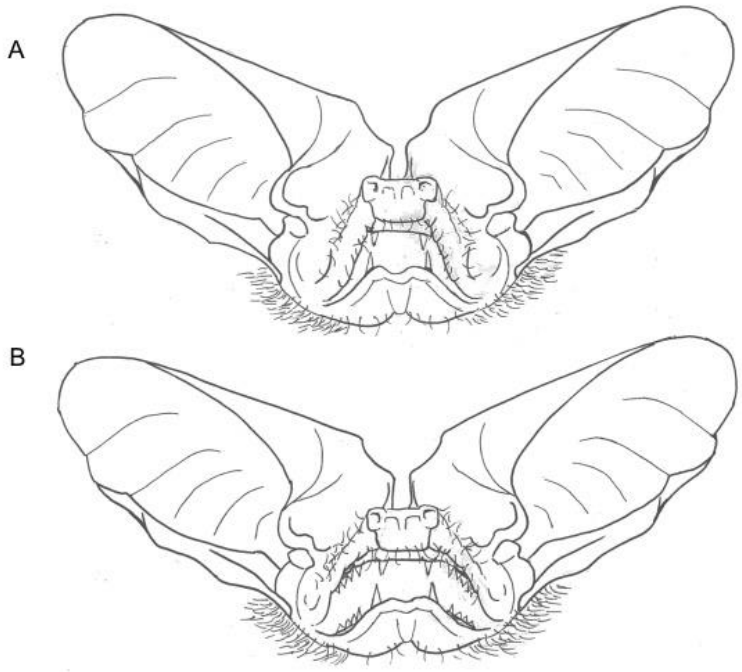


**Figure 7.** Illustrations of *Tadarida* facial configurations. (A) "Nose up" and (B) "Lips up."

Since free-tailed bats emit vocalizations through the mouth, the organization of the nose, lips, and lower jaw creates the aperture shape of the emitter. Sound produced in the larynx travels through the buccal cavity and the beam pattern of outgoing sound is shaped by the size and shape of the aperture (Kinsler et al., 1999). The "nose up" and "lips up" facial configurations I use in this study create small and large emitter apertures, respectively (see open mouths in Figure 7).

## Histology

In order to get an overall picture of the histological properties of this muscle, I used a hematoxylin and eosin stain on frozen sections of the *M. levator labii superioris alaque nasii*, posterior portion. The average cross-sectional area was 1.5mm$^2$ +/- 0.01mm$^2$, and the average cross-sectional fiber density was 2,860 muscle fibers +/-10 fibers per muscle (Figure 8) when I analyzed 8 sections from four bats. Only the posterior portion was used here, as the anterior portion was too small to be sliced and mounted onto slides.

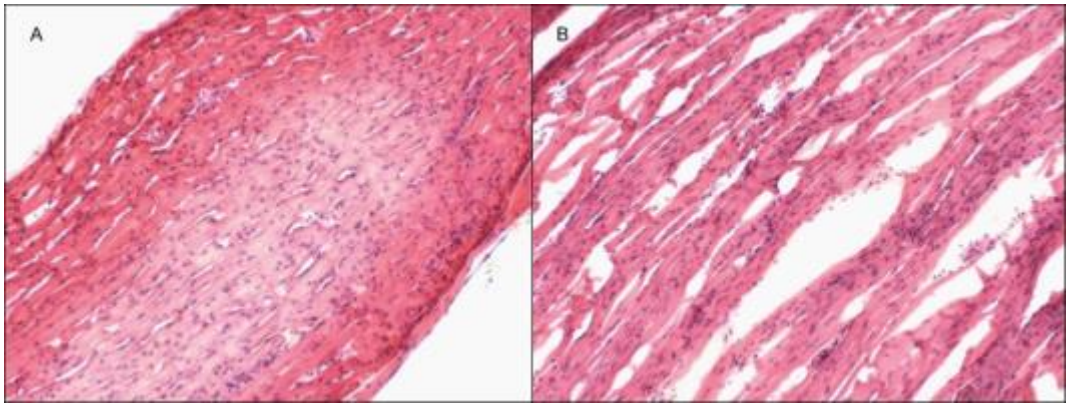


**Figure 8.** Representative fiber organization of the *levator labii superioris alaque nasii*. (A) Cross-section. (B) Longitudinal section.

In order to assess whether the *levator labii superioris alaque nasii* is capable of sustaining activity during echolocation behavior for the duration and at the speed that a flying or hunting bat would require, I assessed the fiber type of this muscle complex using an antibody stain for slow myosin fibers (Figure 9). Slow oxidative, or type I, muscle fibers contract and fatigue slowly, are oxidative, and are characterized by low peak force and the expression of Myosin Heavy Chain isoform I (Armstrong and Phelps, 1984; Rivero et al., 1999). Pectoral muscles were a negative control, as they are known to power the rapid wingbeat during extended periods of flight (Figure 9a). Muscles of the upper leg were used as a positive control as they used mainly for holding the legs in position during stationary hanging and are likely to have a large amount of slow Type I fibers (Figure 9b). Staining of the *levator labii superioris alaque nasii* (Figure 9c) showed that this muscle has a very low number of slow Type I fibers (none were seen here), and in terms of Myosin Heavy Chain isoform 1 expression, is much like the fast-twitch pectoral muscle.

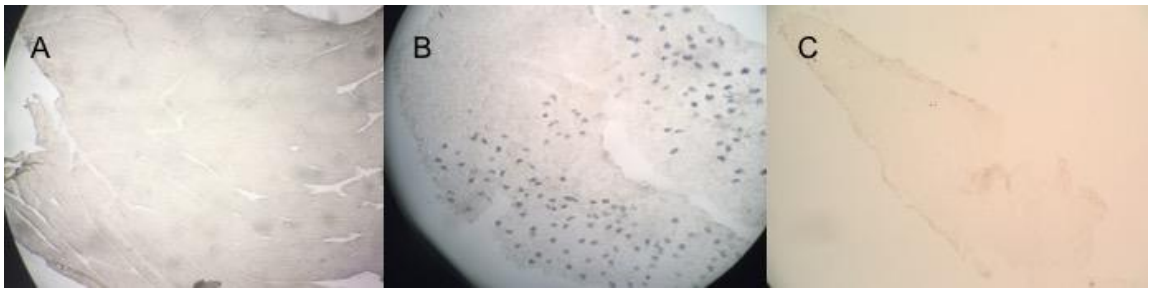


**Figure 9.** Antibody staining for slow-twitch fibers, representative cross-sections. (A) Pectoral muscle. (B) Muscles of the upper leg. (C) Rostral muscle complex, *levator labii superioris alaque nasii.*

## Beam Width Measurements

To assess the beam pattern produced from the two facial configurations produced by differential contraction of the *levator labii superioris alaque nasii,* I measured the sound level 10cm in front of a bat head around a 180 arc in both the horizontal and vertical directions when pure tones were played through the back of the buccal cavity. I played pure tones in 5kHz steps across frequencies within the bats natural echolocation range (25kHz, 30kHz, 35kHz, 40kHz, 45kHz). Here I show the beam patterns from the start and end frequencies of a single echolocation pulse (see Figure 13). At both frequencies, the nose-up position (small emitter aperture) produced a broader and longer beam pattern in the horizontal direction, and a narrower shorter beam pattern in the vertical direction than the beam pattern resulting from the lips-up position (Figures 10 and 11).

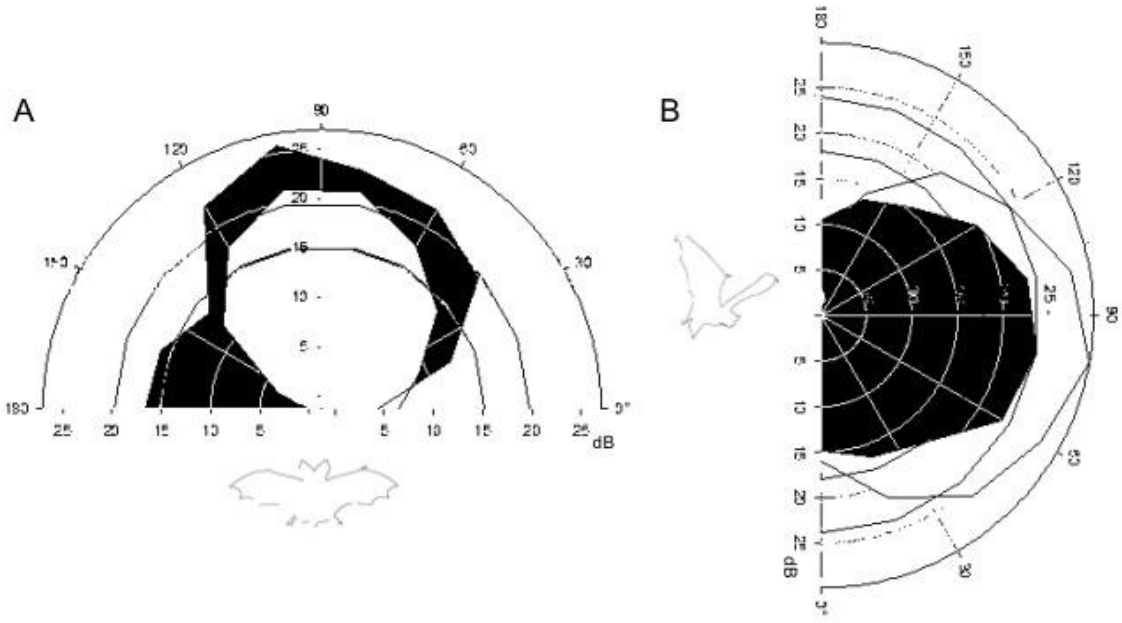


**Figure 10.** Beam projection patterns at 25kHz. Sound intensity (dB) versus radial position. (A) Horizontal projection patterns. (B) Vertical projection patterns. Dark area prodcued from "Nose up" facial configuration, light area from "Lips up" configuration.

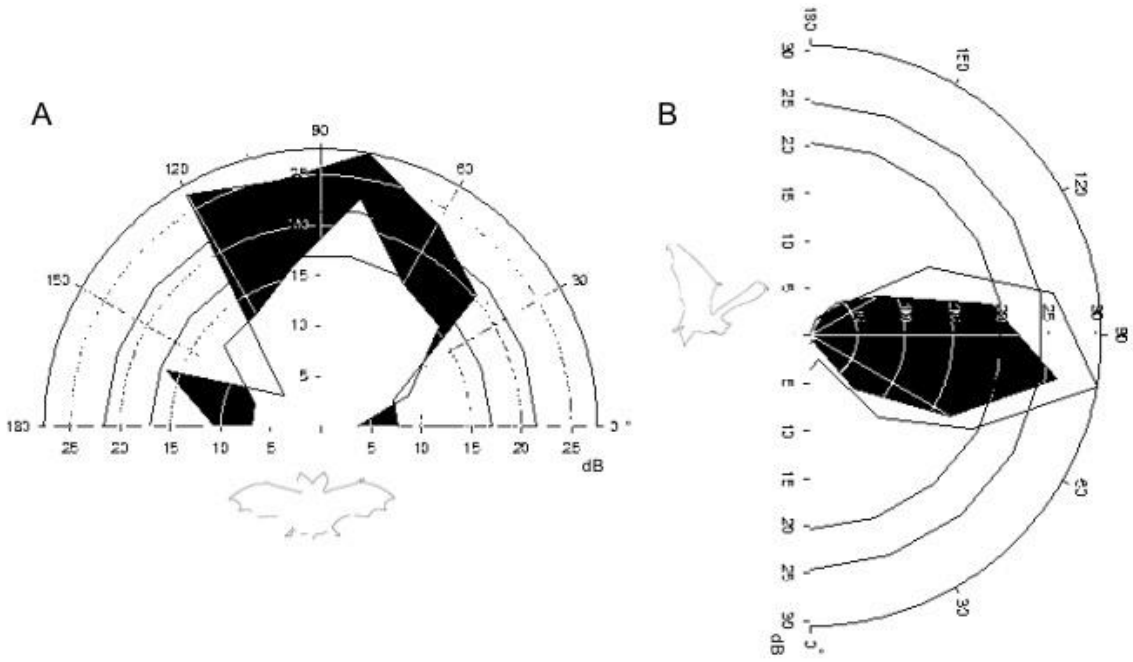


**Figure 11.** Beam projection patterns at 45kHz. Sound intensity (dB) versus radial position. (A) Horizontal projection patterns. (B) Vertical projection patterns. Dark area prodcued from "Nose up" facial configuration, light area from "Lips up" configuration.

## Cortical Stimulation

I used intracortical stimulation and observations of body movements of bats under sedation to locate an area of the motor cortex (M1) where the elements of the bat face are represented. Facial motor cortex is anterior to Bregma and near the rostral border of the cerebral cortex. Much of the facial elements are represented in overlapping areas, and a good deal of the motor area I explored here moved the ears (Figure 12, blue marks). The nose, lips, and jaw had distinct but adjacent areas within M1 (Figure 12, green, yellow, and red marks, respectively).

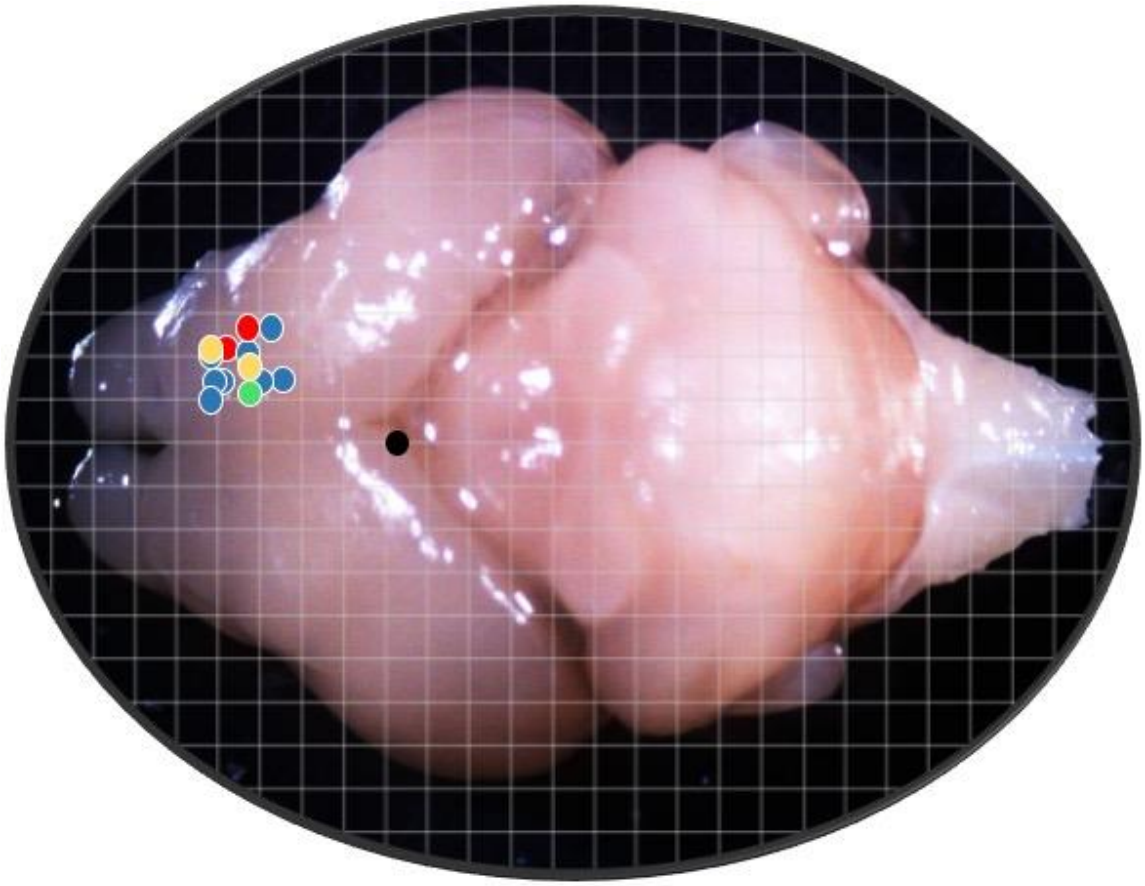

**Figure 12.** Cortical motor map of the facial muscle group in *Tadarida*. Black: Bregma, Blue: ears, Yellow: lips, Red: jaw, Green: nose.

# DISCUSSION

Though bat echolocation beam forming has recently become a popular topic of research, published work provides no mechanism besides a change in gape height (Kounitsky et al., 2015) or a change in peak call frequency (Jakobsen and Surlykke, 2010; Motoi et al., 2017) to account for significant changes in beam pattern in mouth emitting echolocating bats. Vespertillionid bats account for most of the subjects of these studies. Nose-emitting bats seem to use manipulation of features of the face, mainly adjustments in the position of lobes of the nose leaf, to change the size of the echolocation beam, but so far the only conclusive evidence comes from experiments with horseshoe bats alone (Feng et al., 2012; Gupta et al., 2013; Matsuta et al., 2013). Studies outside of the Rhinolophid bats are inconclusive and lack a direct causality between movements of the nose leaf and changes in beam patterns but show that some Phyllostomid bats do have the ability to move parts of the nose leaf (Surlykke et al., 2013; Weinbeer and Kalko, 2007), and that Phyllostomids produce echolocation calls with different beam sizes in different phases of prey pursuit (Linnenschmidt and Wiegrebe, 2016). More definitive evidence is needed to determine if this is a behavior bats are using volitionally and if all nose leaves can be moved to change the beam pattern.

This is the first study to show that bats have a mechanism besides gape to actively adjust the beam pattern. I used high speed video to show for the first time that free-tailed bats lift the nose and lips to emit an echolocation pulse, rather than simply lowering the jaw.

I then used electromyography of the *levator labii superioris alaque nasii* while bats freely emitted echolocation pulses to show that this muscle is active in a precise one to one time-course preceding and during every single echolocation pulse. The muscle was also activated during other behaviors, and is likely not exclusively for echolocation, but the fact that it is always active during echolocation behavior means it is not a trivial factor in production of the pulses.

Free-tailed bats emit echolocation pulses at rates of up to 100 pulses per second (Simmons et al., 1978). A detailed examination of the morphology, innervation, origins and insertions of this muscle along with histological evidence that this muscle has the cellular makeup to support this high-speed repetitive contraction over long periods of time, leads us to conclude that this muscular apparatus is ideally suited for the echolocation behavior such as that used throughout the course of an evening while the animal hunts for prey.

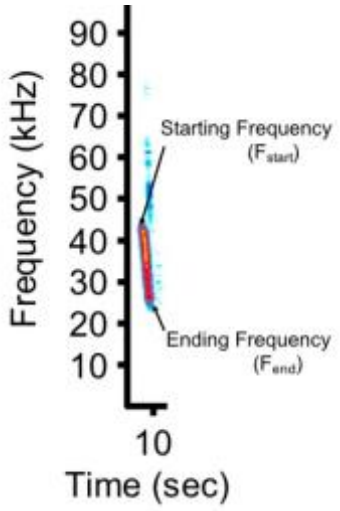


**Figure 13.** Spectrogram of a typical *Tadarida* echolocation pulse.

I used a controlled laboratory approach to show that the positions of the face produced by contractions of the *levator labii superioris alaque nasii* muscular apparatus, 'nose up' and 'lips up,' are sufficient to change the width and height of an outgoing vocalization beam. I used pure tones representative of the start and end frequencies of typical *Tadarida* FM-sweeps (Figure 13). Because higher frequencies have higher rates of atmospheric attenuation they tend to have a different projection pattern than lower frequencies. By getting a view of the range of effects on the entire pulse, I was able to show that simply by changing the position of their nose and upper lips free-tailed bats can broaden or shorten their echolocation pulses in both the horizontal and vertical directions (Figures 10 and 11). Interestingly, the beam pattern changed in unexpected ways – free-tailed bats seem to change their beam patterns by redistributing pulse energy in either the horizontal or vertical plane when energy in the other is reduced.

Based on its location, the *levator labii superioris alaque nasii* may be used simply for snarling or during eating, but this is unlikely given the EMG results I show here and the morphological and histological specializations of the muscle for long-term ballistic action. The *levator labii superioris alaque nasii* is very likely also used during those behaviors, but I argue here that beam forming during echolocation, not snarling or chewing, is its main function. This is the first time a muscle of the face, besides muscles of the jaw to make crude changes in gape height, has been shown to actively change acoustic properties of a vocalization in any mammal besides humans.

This study is also the first to make use of ICMS to make a motor map in bats and the first to show cortical control of a face muscle for supralaryngeal vocal control in a mammal other than a primate. The ICMS technique has been widely used in primates (Huerta et al., 1986; Luppino et al., 1991; Mitz and Wise, 1987), and larger mammals like cats (Asanuma et al., 1976; Asanuma and Ward, 1971; Ronner et al., 1981), but Tennant *et al.* (Tennant et al., 2011) was the first to use ICMS in a small mammal, such as a mouse, to produce a similar map to the one I present in Figure 12. I was able to modify their approach and use small stimulations *in vivo* to identify areas of the bat motor cortex which move certain areas of the face. This map is still preliminary, but provides evidence that the *levator labii superioris alaque nasii* muscular apparatus is under cortical control, and may be used volitionally by the bat to modify beam pattern according to auditory feedback during quickly changing conditions such as those during flight through varying environments, or with large groups of conspecifics, or while hunting.

The goal of this study is to provide controlled, laboratory based groundwork for future experiments exploring beam patterning in free-tailed bats. Future work will explore beam patterns of the entire vocal repertoire of the free-tailed bat, including echolocation pulses and social calls, will measure beam pattern from flying and stationary bats, will compare beam pattern results from bats in the wild versus bats in the lab, and will measure *levator labii superioris alaque nasii* EMGs in a flying bat and capture the facial changes simultaneously.

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