

Echolocation Behavior and Foraging Strategies of the FM Bat *Natalus tumidirostris*

Echolocation Behavior and Foraging Strategies of the FM Bat

Natalus tumidirostris

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”Wahrhaft kosmische Ansichten sind erst Folge der Beobachtung und ideeller Combination, Folge eines lange dauernden Contacts der Menschheit mit der Außenwelt; auch sind sie nicht das Werk eines einzigen Volkes, sie sind die Frucht gegenseitiger Mitteilung eines [großen] Völkerverkehrs.”

”True cosmic views are the result of observation and the combination of ideas, and of a long and continued contact of humankind with the external world; they are also not the work of single people, but the fruits yielded by reciprocal and [great] exchange between peoples.”

- Alexander von Humboldt -

Acknowledgments

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As one can see, the work comprised in the following pages is by no means my lone undertaking. Therefore, I took the liberty of writing this text using the words "we" and "our" instead of "I" or "my"; to honor and highlight all of you and your input in this work. From the bottom of my heart, thank you all.

Abstract

Bats utilize echolocation to navigate and forage in diverse habitats, facing distinct challenges that vary with environmental complexity. Particularly challenging is the detection of fluttering prey in highly cluttered environments, a capability that has never been conclusively demonstrated in frequency modulating (FM) bats. This is why this dissertation explores the echolocation behavior and foraging strategies of the FM bat *Natalus tumidirostris*, a species about which little is known. We investigated its echolocation and foraging behavior in a semi-natural environment within a flight tent, assessed how its call characteristics enable it to locate prey in cluttered environments, and examined its dietary preferences using both behavioral experiments and metabarcoding techniques.

Initially, we characterized the echolocation behavior of *N. tumidirostris*, emphasizing its unique method of continuous sampling during the search phase. This species emits faint, short (1.2 ms), broadband, high-frequency FM calls with brief pulse intervals of 26 ms. To our knowledge, no other bat species continuously produces calls without grouping them, maintaining such a high call emission rate of 38 Hz over extended periods during the search phase. This echolocation strategy, combined with morphological adaptations like low wing loading and a low aspect ratio, equips *N. tumidirostris* for highly maneuverable flight in cluttered spaces, indicating a specialized evolutionary adaptation for hunting in narrow space.

Our research provides novel evidence that *N. tumidirostris* uses flutter information contained in the echoes from fluttering insects to detect and possibly also to characterize prey. In behavioral experiments, *N. tumidirostris* showed a clear preference for fluttering over non fluttering moths. This preference was also demonstrated under behaviorally relevant scenarios where the bats were able to detect fluttering moths as close as 10 cm from dense clutter. Furthermore, we also observed that *N. tumidirostris* approached a rotating propeller that simulated a fluttering insect. To our knowledge, this is the first study to conclusively show that a FM bat uses flutter information contained in the echoes

from the insects it hunts; an ability so far only shown for constant frequency (CF) bats. Moreover, we explore how the echolocation behavior of *N. tumidirostris* is suited for detecting insect flutter: our Monte Carlo simulations indicate that the species' continuous emission of calls during the search phase should allow it to receive approximately four acoustic glint echoes from a moth per second. We discuss how *N. tumidirostris* could extract flutter information from these echoes, either by perceiving sudden amplitude increases within a dominant frequency band or distinct spectral characteristics of the glint echoes, leading to the hypothesis of an insect fluttering. Following detection, *N. tumidirostris* shifts its echolocation behavior to an inspection phase, where the pulse intervals are reduced to an average of 18 ms. We discuss how *N. tumidirostris* should be capable of estimating the insect's flutter frequency during this phase by means of a simple integrator mechanism that counts the number of detected glints per unit of time or by Compressive Sensing, thus enabling it to effectively characterize its prey before attempting a catch. Following this inspection phase, *N. tumidirostris* proceeded to the final approach towards the insect, where the pulse interval was reduced to values around 6 ms shortly before prey capture.

Finally, our diet analysis confirms Lepidoptera as the predominant order in the diet of *N. tumidirostris*. The metabarcoding analysis also reveals a very large number of sequences belonging to the Tachinidae family, which are flies that parasitize on insect larvae (especially Lepidoptera). Since adult Tachinids are diurnal and so do not overlap in activity with bats, we conclude that *N. tumidirostris* must be consuming caterpillars infested by larvae of Tachinids. The presence of non-fluttering prey such as caterpillars as well as a few number of spiders in the diet of *N. tumidirostris* suggests this bat also uses active gleaning as an additional foraging strategy to flutter detection to hunt for insects in highly cluttered environments.

Kurzfassung

Fledermäuse nutzen Echoortung zur Orientierung und Nahrungssuche in verschiedenen Lebensräumen. Sie begegnen dabei unterschiedlichen Herausforderungen, die von der Komplexität der Umgebung abhängen. Besonders herausfordernd ist die Detektion von flatternder Beute bei dichter Vegetation; eine Fähigkeit, die bei frequenzmodulierenden (FM) Fledermäusen noch nie schlüssig nachgewiesen wurde. Aus diesem Grund untersucht diese Dissertation das Echoortungsverhalten und die Jagdstrategien der wenig erforschten FM-Fledermausart *Natalus tumidirostris*. In Verhaltensexperimenten im Flugzelt unter seminatürlichen Bedingungen untersuchten wir das Echoortungs- und Jagdverhalten beim Fang von Insekten und diskutieren, wie das Echoortungssystem dieser Art angepasst ist, Beute im narrow space mit Echoortung zu detektieren und zu evaluieren. Des Weiteren führten wir Kotanalysen mittels Metabarcoding durch, um das Beutespektrum von *N. tumidirostris* zu bestimmen.

Im Suchflug sendete *N. tumidirostris* kontinuierlich sehr leise, kurze (1.2 ms), breitbandige, hochfrequente FM-Laute mit einem sehr konstanten Pulsintervall von durchschnittlich 26 ms aus. Nach unserem Wissen produziert keine andere bisher beschriebene Fledermausart im Suchflug kontinuierlich Rufe mit einer Rufrate von 38 Hz, ohne die Laute zu gruppieren. Diese Echoortungsstrategie, in Kombination mit morphologischen Anpassungen wie einem sehr niedrigen wing loading und sehr niedrigen aspect ratio, welche diese Art zu einem äußerst manövrierfähigem Flug im hindernisreichen Räumen befähigen, deutet darauf hin, dass *N. tumidirostris* für die Jagd im narrow space angepasst ist.

Unsere Verhaltensuntersuchungen zeigen darüber hinaus, dass *N. tumidirostris* die in den Echos flatternder Insekten enthaltenen Flatterinformation nutzt, um Beute zu detektieren und möglicherweise auch zu charakterisieren. In einem Verhaltensexperiment zeigte *N. tumidirostris* eine klare Präferenz für flatternde gegenüber nicht flatternden Motten. In einem weiteren Verhaltensexperiment, bei dem Motten 10 cm vor dichter Ve-

getation präsentiert wurden, fingen die Tiere die Motten nur, wenn sie flatterten. Außerdem flog *N. tumidirostris* einen rotierenden Propeller an, welcher den Flügelschlag eines Insekts simuliert. Unseres Wissens ist dies die erste Studie, die schlüssig zeigt, dass eine FM-Fledermaus die in den Echos der Insekten enthaltene Flatterinformation nutzt; eine Fähigkeit, die bisher nur für CF-Fledermäuse (*constant frequency*) nachgewiesen wurde. Darüber hinaus untersuchten wir, wie sich das Echoortungsverhalten von *N. tumidirostris* für die Erkennung von Flatterinformation eignet: Unsere Monte-Carlo-Simulationen zeigten, dass die Art durch die kontinuierliche Aussendung von Rufen während der Suchphase in der Lage sein sollte, von einer Motte pro Sekunde etwa vier Echos mit glint zu erhalten. In einem einzelnen Echo mit Glint ist Flatterinformation in Form einer plötzlichen Amplitudenerhöhung innerhalb eines begrenzten Frequenzbandes sowie durch die spektralen Eigenschaften des Glints kodiert. Wir gehen davon aus, dass die Information in einem einzelnen Echo für die Hypothese ausreicht, dass das Echo von einem flatternden Insekt kommt. Nach der Detektion wechselte *N. tumidirostris* im Echoortungsverhalten vom Suchflug zur Inspektionsphase, die im Mittel 0.6 s dauerte und in der die Pulsintervalle auf durchschnittlich 18 ms reduziert wurden. Wir diskutieren, wie *N. tumidirostris* in der Lage sein könnte, die Flatterfrequenz des Insekts während dieser Phase abzuschätzen, entweder durch einen einfachen Integrationsmechanismus, der die Anzahl der erkannten glints pro Zeiteinheit zählt, oder durch Compressive Sensing. An die Inspektionsphase schloss sich die finale Annäherung an das Insekt an. Dabei wurde der Lautabstand auf Werte um die 6 ms kurz vor dem Fang abgesenkt.

Die Kotanalyse mit Metabarcoding ergab, dass der Hauptbestandteil der Nahrung von *N. tumidirostris* aus Insekten der Ordnung Lepidoptera besteht. Außerdem ergab diese Analyse einen hohen Anteil an DNA von Fliegen aus der Familie der Tachinidae. Da diese Fliegen Insektenlarven, insbesondere aus der Ordnung der Lepidoptera, parasitieren und die adulten Fliegen tagaktiv sind, gehen wir davon aus, dass *N. tumidirostris* Raupen konsumiert, die von Tachiniden befallen sind. Die Anwesenheit von nicht flatternder Beute wie Raupen und eine reduzierte Anzahl von Spinnen in der Nahrung von *N. tumidirostris* deutet darauf hin, dass diese Fledermaus neben Flatterdetektion auch das "aktive gleaning" als Jagdstrategie nutzt, um Insekten im narrow space zu jagen.

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Chapter 1

Introduction

Bats, constituting the order Chiroptera, showcase a remarkable evolutionary journey within the animal tree of life in terms of their biology and ecology. The diversity found among bat species represents an “unparalleled exhibition of variations on the mammalian theme” ([Altringham \(2001\)](#)), boasting over 1400 species that occupy multiple ecological niches across the globe. Representing the second most diverse group of mammals after rodents, bats have conquered all continents except Antarctica, and are sometimes the sole mammalian inhabitants of isolated islands ([Altringham \(2001\)](#)). This evolutionary success is mostly granted due to two main adaptations: their ability to fly and their ability to use echolocation. It is this unique blend of traits that have enabled bats to conquer the night sky and exploit a vast number of ecological niches and habitats: from the dense canopies of tropical forests to the vastness of arid deserts. As the only mammals capable of sustained flight, bats play a crucial role in ecosystem services, including pollination, seed dispersal, and insect population control. Particularly, the evolution of echolocation is not only distinctive for bats, but it has also intrigued scientists since its discovery ([Griffin \(1944\)](#)), laying a fertile ground for extensive research into its underlying mechanisms, but also into bat behavior, ecology, and conservation.

Echolocation, or the ability to navigate and forage by emitting sounds and listening for the echoes returned from objects in the environment, represents an **active** sensory system, distinct from the passive sensory modalities humans primarily use for navigation. We as humans hear, see, and feel what is out there in the environment that surrounds us. All our sensory input is acquired passively by our sensory organs. In contrast, bats emit sound waves that travel in space, collide with surrounding objects, and then bounce back to their ears as echoes (see Fig. [1.1](#)). By analyzing and comparing the time and

spectral parameters between the emitted calls and their returning echoes, bats create an “acoustic picture” and an “acoustic flow” of the outside world (Surlykke *et al.* (2016)). Echolocation can represent the outside world so accurately, that some insectivorous bats are even capable of predicting an insect’s flight trajectory in a complex three-dimensional space and position themselves accordingly for interception (Kalko (1995); Salles *et al.* (2020)).

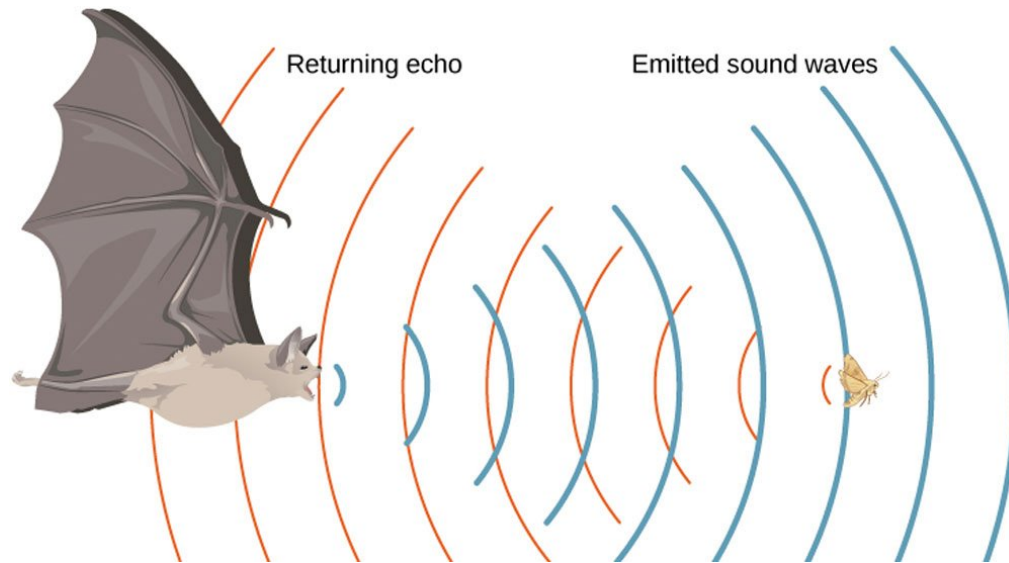


Figure 1.1: Echolocation is an active sensory system. This figure illustrates the fundamental mechanism of bat echolocation, depicting a bat in flight actively emitting sound waves toward a flying insect. The sound waves, represented by blue concentric arcs, travel through the air and encounter the insect, at which point some of the sound energy is reflected back towards the bat as echoes, visualized here as red concentric arcs. This active sensory system allows the bat to analyze these returning echoes to determine the location, velocity, and even some aspects of the size or shape of the prey. The clear distinction between emitted sound waves and returning echoes demonstrates the principle of how bats interpret time delays, monaural and binaural cues, spectral information, as well as other echo statistics (Yovel *et al.* (2009); Yovel *et al.* (2011)), to create an acoustic image of their environment. (Extracted from <https://transom.org/other-pieces/mist-nets-long-ears> on April 9th, 2024.)

Bats modify their echolocation behavior as they transition through the different phases of prey detection and capture (see Simmons *et al.* (1979)). Although these phases may vary depending on the echolocation behavior required to hunt in a particular habitat, or the particular prey a bat is hunting, there is a general structure in the echolocation behav-

ior for bats hunting aerial prey: during the **search phase**, bats usually emit rather longer, and lower frequency echolocation calls emitted in the rhythm of every wing beat to detect prey from a distance. Once they detect a prey, bats switch to the **approach phase**, where the calls become shorter, more frequent, and broaden in bandwidth, facilitating the precise localization of prey (Simmons *et al.* (1979)). Finally, the approach ends with a **terminal phase** (or buzz phase), which occurs just before capture, the call rate dramatically increases, and call duration shortens further, providing rapid updates on the prey's position for successful interception (Kalko (1995); see Fig. 1.2).

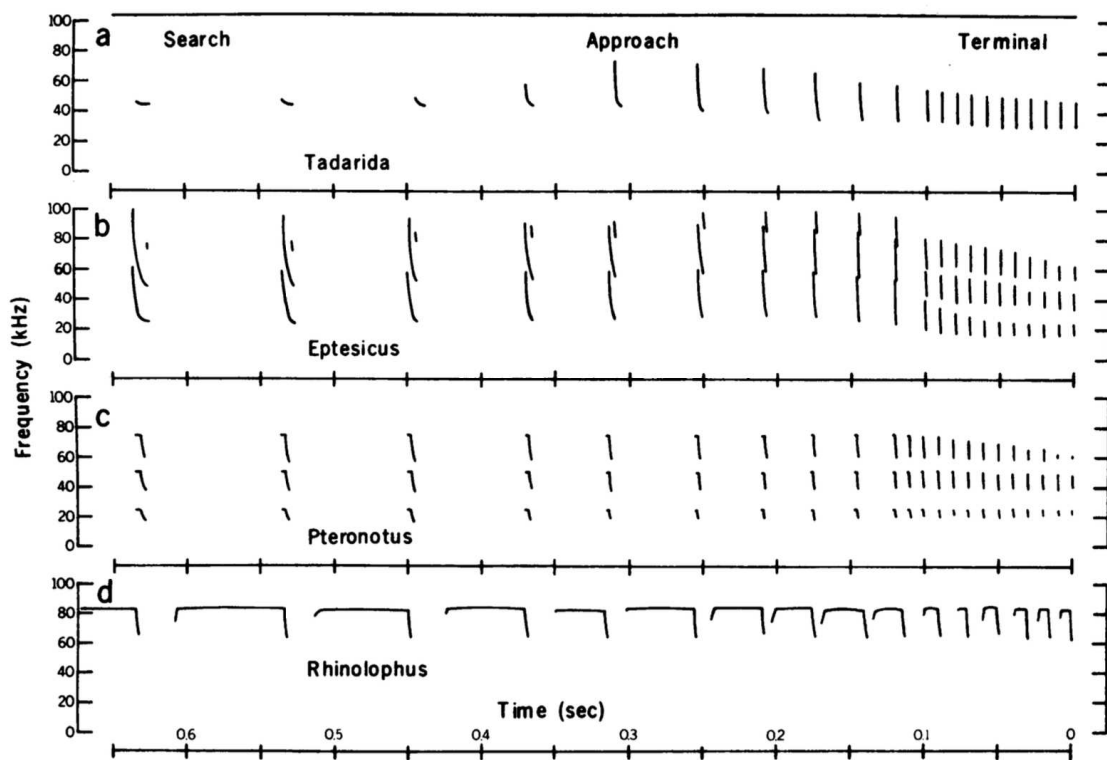


Figure 1.2: **Phases of prey detection and capture in echolocating bats hunting for aerial prey.** This figure presents sonar signal spectrograms of four bat species—*Tadarida brasiliensis*, *Eptesicus fuscus*, *Pteronotus personatus*, and *Rhinolophus ferrumequinum*—during the search and approach phases of a typical prey pursuit sequence, completed in less than one second. Note the time scale is shown in reverse, leading up to the moment of prey capture. Also, note the variations in call length and frequency, as well as call emission rate, across the different phases. Taken from Simmons *et al.* (1979).

1.1 Bat Guilds

Given the sophisticated nature of echolocation, it's not surprising that bats have capitalized on this ability to exploit a broad spectrum of ecological niches. This adaptive diversity in echolocation usage has been categorized by scientists into so called **bat guilds** (Schnitzler and Kalko (2001); Schnitzler *et al.* (2003); Denzinger and Schnitzler (2013); Denzinger *et al.* (2016); Denzinger *et al.* (2018)), which represent a framework to group bat species based on their specific foraging habitat and echolocation strategy. Despite their varied evolutionary backgrounds, bats of the same guild share many behavioral and morphological adaptations that arise from engaging in similar tasks, particularly in their modes of foraging and the habitat type in which they search for prey. Here, the distance between bat and background and between prey and background are the most relevant ecological constraints that shaped the sensory and motor systems of bats (Schnitzler *et al.* (2003)). By grouping species into guilds, researchers have been able to better understand the structure of bat communities and the ecological niches they occupy. Denzinger and Schnitzler and their collaborators use two primary factors to define bat guilds: the bats' foraging habitat—categorized according to the clutter conditions into open space, edge space, and narrow space (see Fig. 1.3)— and their echolocation behavior while foraging, such as aerial hunting, trawling, gleaning (both active and passive forms) and flutter detection. It is also important to highlight that the bat guilds are strictly defined based on the echolocation behavior during the search phase (Schnitzler *et al.* (2003)). This is due to the fact that during the approach phase, all bats have to perform a rather similar task, regardless of the echolocation strategy or environment they forage on.

Next, we will briefly dive into the bat guilds regarding insectivorous bats, as it is essential to understand the problem we will be confronted throughout this thesis (also, please refer to Fig. 1.3 for a visual representation of the habitat types of each guild and to Fig. 2 in Denzinger and Schnitzler (2013) for an overview of the acoustic scenes a bat encounters in those habitats):

- **Open Space Aerial Foragers**, belonging to families such as Rhinopomatidae, Emballonuridae, Vespertilionidae, and Molossidae, mainly forage in open spaces where they usually locate dispersed prey. Open space foragers possess “small pointed wings with high aspect ratio which give high agility. Such [wings are] adapted for a fast aerial hawking flight” Denzinger and Schnitzler (2013). Back-

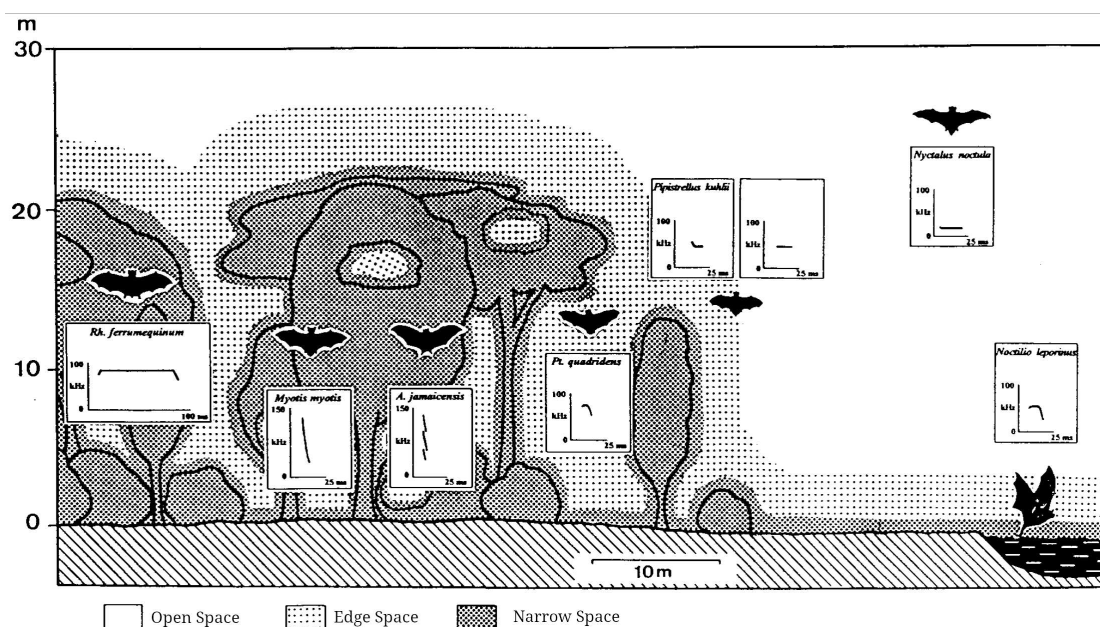


Figure 1.3: Bat Guilds and their echolocation call characteristics. This figure outlines the foraging spaces of bats based on clutter level, with silhouettes and echolocation calls of exemplar species. Bats in open space hunt prey away from any vegetation or ground interference. In edge space, they pursue insects near obstacles like vegetation edges or water surfaces. Bats foraging in narrow space hunt prey between or directly on vegetation. Generally, as the level of clutter increases, bats tend to emit shorter calls with broader bandwidth (except for the CF bat *R. ferrumequinum*, see Section 1.4). Edited from [Schnitzler and Kalko \(2001\)](#).

ground echoes are so far away that bats do not react to them. They emit narrow-band, shallowly modulated search signals lasting between 8 to 25 ms at frequencies below 30 kHz, thus optimizing prey detection over large distances. This echolocation strategy not only boosts the detection likelihood by concentrating signal energy within the auditory system's neuronal filters but also enhances the detection of acoustic glints from fluttering prey wings, which are sudden amplitude increases in a returning echo after perpendicularly hitting the moving wing ([Schnitzler \(1987\)](#)). Pulse intervals are often multiples of the bat's wingbeat rate as they do not emit a call every wingbeat cycle. These foragers achieve remarkable detection ranges, with average source levels spanning 104 to 111 dB SPL at 1 m, enabling species like *Nyctalus noctula* to detect prey up to distances ranging between 3.5 m and 10 m ([Stilz and Schnitzler \(2012\)](#)), or likely even higher under optimal conditions

- (Holderied and Von Helversen (2003); Jung *et al.* (2007); Jakobsen *et al.* (2013)). Their echolocation also aids in navigation and habitat recognition, allowing for the identification of environmental features like forest edges and water surfaces from up to 54 m away (Stilz and Schnitzler (2012)). Upon prey detection, they engage in an approach sequence, marked by a shift to higher bandwidths and shorter pulse durations and intervals with calls arranged in groups, culminating in a terminal buzz phase where pulse interval in buzz I drops to about 6 ms and is kept constant at this value in buzz II.
- **Edge Space Aerial Foragers** are usually encompassed by families such as Emballonuridae, Mormoopidae, Vespertilionidae, and Molossidae. Their wings have “average aspect ratios and wing loadings and rounded tips [and] are adapted for slow inexpensive flight in the vicinity of background objects” Denzinger and Schnitzler (2013). The members of this guild are adapted to navigate the semi-cluttered environments of edge spaces in which they must detect prey where obstacles are present but not pervasive, such as along the edges of vegetation or water bodies or in forest gaps. Their signal repertoire includes mixed search signals, blending shallowly modulated narrowband components with broadband, steeply modulated components for enhanced localization and obstacle avoidance, across intermediate distances of 1.5 to 7 m. These signals, ranging in duration from 3 to 10 ms and in frequency from 30 to 60 kHz for the narrowband part, are emitted in synchrony with each wingbeat or in pairs when in close proximity to clutter, supporting precise maneuvering (Stilz and Schnitzler (2012); Schaub and Schnitzler (2007)). The source levels for these bats are slightly lower than their open space counterparts, lying between 101 and 107 dB SPL at 1 m, optimizing their echolocation for intermediate ranges (Holderied and Von Helversen (2003); Surlykke and Kalko (2008)). Edge space foragers modify their echolocation behavior in response to background targets. As they approach the background, they adjust their echolocation calls, shortening duration and broadening bandwidth to maintain an overlap-free window for echo processing. This allows them to better perceive the acoustic environment where insect prey can be detected and also gather detailed localization cues for precise navigation around obstacles.

- **Edge Space Trawling Foragers**, represented across the Vespertilionidae, Noctil-

ionidae, and Phyllostomidae families, excel in skimming just above water surfaces to hunt insects or fish. Their wings are long and have “a higher aspect ratio than most other bats but have only a medium wing loading. Such a wing is adapted for economic flight above water surfaces that also allows slow flight” [Denzinger and Schnitzler \(2013\)](#). These bats employ signals with both steeply and shallowly modulated components, typically spanning 3–7 ms in duration with frequencies tailored between 30 and 60 kHz. Their echolocation scene is similar to that of other edge space foragers as most of the sound waves are reflected away from the water surface. Only echoes reflecting from 90° directly below the bat reflect back and encode the flight height above water. Prey echoes from insects drifting on or flying above the water surface arrive later, followed by echoes from the shore area if the bat flies close to it. Challenges arise in turbulent waters or when debris masks the echoes of prey, complicating detection ([Rydell et al. \(1999\)](#); [Boonman et al. \(1998\)](#)). The intensity of their calls varies based on their proximity to background elements, indicating their flexible approach to foraging, with source levels ranging notably between species (from about 100 dB SPL in *Myotis daubentoni* cite [Surlykke et al. \(2009\)](#)) to around 116 dB SPL in *Noctilio leporinus*, measured at 1 m (cite [Surlykke and Kalko \(2008\)](#)). Morphologically, these edge space trawling foragers are equipped with adaptations like specialized hind legs and sharp claws for piscivorous species, emphasizing their unique niche within aquatic environments (e.g., [Thompson and Fenton \(1982\)](#); [Schnitzler et al. \(1994\)](#); [Weinbeer and Kalko \(2007\)](#); [Surlykke and Kalko \(2008\)](#)). Similar to open space foragers, when detecting prey edge space foragers switch in their echolocation behavior from search to approach, by arranging calls in groups and by a continuous reduction of pulse duration and pulse interval. The approach ends like in all aerial foragers with a buzz.

- **Narrow Space Foragers** are distinguished by their foraging within cluttered environments, where echoes from food items overlap or interfere with background echoes ([Denzinger and Schnitzler \(2013\)](#)). Their wings with low aspect ratio and wing loading allow for a high maneuverable flight in cluttered spaces. This category is further subdivided based on how bats detect and capture their prey, but before describing those in further detail, it is important to understand the masking problem bats face when hunting for insects in such narrow space environments.

1.2 The Masking Problem

In the cluttered environments where narrow-space foraging bats hunt, they face significant auditory challenges, specifically forward- and backward-masking effects. Forward masking occurs when the bat's own emitted echolocation signal temporally overlaps with or is too close in time to echoes returning from prey, thus “obscuring” these crucial echoes. On the other hand, backward masking is caused by echoes from background objects (clutter echoes) that temporally overlap with or are near in time to the echoes from prey, making it difficult for bats to discern vital prey echoes from background clutter. These masking effects can significantly hinder a bat's ability to detect and locate an insect prey, as bats rely on unmasked echo signals from the prey to be able to track it. In other words, “information about the prey [is] available to [bats] only in an overlap-free window within which prey echoes overlap neither the emitted signal nor echoes from background clutter” (Schnitzler and Kalko (2001); Schnitzler and Kalko (1998); see Fig. 1.4).

To counteract masking issues, bats can modulate their echolocation behavior. As previously mentioned, edge space foragers reduce the duration of their calls when hunting nearer to clutter, a tactic that directly expands the overlap-free window (see Fig. 1.4). For instance, a decrease in call duration by just 1 ms can widen this window by 0.34 m (Denzinger and Schnitzler (2013)), significantly enhancing the bat's ability to detect prey echoes without interference from its own signal or from clutter echoes. However, narrow space foragers that hunt for insects deep within cluttered spaces, where prey echoes overlap or interfere with background echoes, need to implement even more sophisticated strategies.

1.3 Narrow Space Foragers' Guilds

Narrow space Passive Gleaning Foragers, encompassing members from the Phyllostomidae, Megadermatidae, Nycteridae, and Vespertilionidae families, circumvent the challenge of clutter by relying primarily on non-echolocation cues—such as the sounds made by prey—to detect and locate their prey (Schmidt *et al.* (2000); Arlettaz *et al.* (2001); Goerlitz *et al.* (2008); Page and Ryan (2008)). Vision may complement these auditory cues under certain conditions, enhancing their prey detection capabilities (Bell (1985);

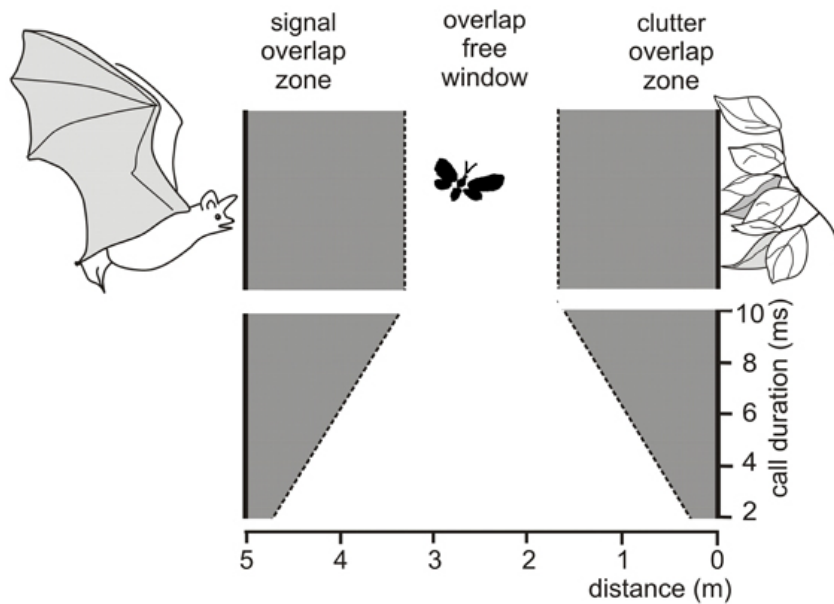


Figure 1.4: **Bats adjust call duration to avoid masking effects.** When the echo of an insect overlaps with either the bat's own call or with echoes from clutter (gray areas), the perception of the prey item is compromised. To reduce such masking effects, bats can modulate the duration of their calls. Assuming a standard sound speed of 340 m/s , a 1 ms reduction in call duration corresponds to a 0.34 m increase in the overlap-free window (white area). Taken from [Denzinger and Schnitzler \(2013\)](#).

[Eklöf and Jones \(2003\)](#)). Upon detecting these cues, the bats employ echolocation primarily for navigation towards the site of the prey, subsequently relying on tactile and olfactory senses for final prey localization and capture. The echolocation behavior in this guild is characterized by short, broadband signals emitted at low source levels, often in groups that align with the bats' wingbeats. The call structure is well-suited for spatial orientation, obstacle avoidance, and biotope recognition, facilitating an increased signal rate and grouping as the bat approaches its target ([Denzinger and Schnitzler \(2013\)](#)). The approach sequence concludes with a terminal group consisting of a few signals only, setting them apart from aerial foragers.

Narrow space Active Gleaning Foragers, exemplified by species like *Micronycteris microtis*, demonstrate a more echolocation-centric approach, which allows them to discern stationary prey concealed among or on background objects, such as insects like dragonflies resting on leaves ([Geipel et al. \(2013\)](#); [Geipel et al. \(2019\)](#)). By employing a methodical scanning process, where each leaf is investigated from an oblique angle

above, *M. microtis* can rapidly assess if there are any insects resting on a leaf. Echoes from unoccupied leaves return a pattern with distinct echoes from the leafs' frontal and posterior edges, followed by further clutter echoes from beyond, while sound waves that hit the surface of the leaf are reflected away. In contrast, prey-occupied leafs yield an additional distinctive echo from the prey item between the leafs' edge echoes (Geipel *et al.* (2013); Denzinger and Schnitzler (2013)). This echolocation mechanism “resembles the echolocation scene in the trawling mode, but on a micro time scale” (Denzinger and Schnitzler (2013)). When foraging for stationary prey, *M. microtis* emit multi-harmonic, ultra-short (0.2 ms), broadband, and high-frequency calls with low source levels, organized into groups. The very low source level and the high frequencies of the calls are a strategy for clutter rejection, as such calls travel only short distances and thus reduces the clutter from objects further away (Jakobsen *et al.* (2013)). As they finalize their approach towards the prey, the echolocation pattern culminates in a group of 3–5 signals, notably absent from the distinct buzz phase that is typically observed in aerial foraging strategies.

Narrow Space Flutter detecting foragers, on the other hand, exhibit a highly specialized form of echolocation that allows them to exploit a unique acoustic niche. Members of this guild - which comprise all Rhinolophidae, Hipposideridae and the Mormoopid *Pteronotus parnellii* - are able to discern flutter information within the returning echoes of their calls by means of crucial adaptations in both their transmitter and receiver systems (for an extensive review, see Schnitzler and Denzinger (2011)). These adaptations enable them to effectively overcome the masking problem in narrow space.

1.4 Decoding insect flutter information using CF echolocation

The two primary adaptations of the transmitter system are the increase of duty cycle and **Doppler shift compensation (DSC)**. These bats emit 10-80 ms long echolocation calls with a characteristic **constant frequency (CF)** component. The duty cycle, a measure of the percentage of time filled with calls, is notably increased when these bats encounter echoes with flutter information, such as from the wing beats of prey. This adaptation enables an enhanced detection of '**acoustic glints**'—distinct, momentary increases in amplitude (up to 20 dB) and spectral shifts (up to 2 kHz) in the echo in the rhythm of the insect's wing beat caused by the wings of fluttering insects when hit perpendicularly by

the bat's sonar beam (Schnitzler and Denzinger (2011)). These glints provide dynamic and precise auditory cues that reflect the aspect angle, wingbeat frequency, and the physical characteristics of the insect, allowing bats to localize, track and classify their prey effectively (Schnitzler (1987); Schnitzler and Flieger (1983); Schuller (1984); Kober (1988); Ostwald *et al.* (1988); Kober and Schnitzler (1990)). CF bats can increase their duty cycle by lengthening the emitted signals, by increasing the number of signals, or by a combination of both (Schnitzler and Denzinger (2011)). The duty cycle in CF bats can reach up to 60% in species like *Rhinolophus ferrumequinum*, thus allowing them to monitor multiple glints within a single echolocation call (Schnitzler and Flieger (1983); Neuweiler *et al.* (1987); Neuweiler (1990)).

The other key adaptation of the transmitter system in CF bats, Doppler shift compensation, represents an intricate feedback mechanism that adjusts the frequency of emitted echolocation calls to stabilize the frequency of returning echoes. Doppler shift occurs when the frequency of a sound wave increases or decreases due to the relative motion between the source of the sound and the observer. In the context of bats, this shift happens as they and their targets (whether moving prey or stationary objects) approach or move away from each other, affecting the frequency of the echoes received by the bats. DSC allows CF bats to finely alter the pitch of their calls in response to their own velocity changes, ensuring that the echoes return at a constant, optimal frequency known as the 'reference frequency'. The audio-vocal control mechanisms in CF bats has been subject of thorough study (Metzner (1993); Smotherman and Metzner (2003); Schuller and Moss (2004)).

But why do CF bats possess such an advanced mechanism such as DSC, that ensures the frequency of their returning echoes stays within a very specific and narrow range? The answer of this question takes us to the adaptations of the receiver system, which are crucial for accurately processing and evaluating the flutter information contained in the echoes. One key adaptation involves neurons that are sharply tuned to the second harmonic of the CF component, aligning with the reference frequency utilized during DSC (Pollak *et al.* (1972); Suga *et al.* (1974)). This tuning is 25 times sharper than in any FM bat, with Q_{10dB} values (best frequency divided by bandwidth of the tuning curve; 10 dB above minimal threshold) of up to 500, compared to 20 in FM bats. Such a sharp tuning of neurons in the basilar membrane shows how well optimized the bat's auditory system is for detecting and processing any slight modulations contained in the

echoes from their prey. Also, the audiograms of these bats display a sharp minimum at or near the reference frequency, with a distinct maximum a few kHz below, enhancing the detection of Doppler-shifted echoes, while avoiding self-stimulation from the bat's own louder emissions (Neuweiler (1970); Suga and Jen (1977)).

A second and very prominent adaptation is a specialized area within the cochlea, described as an '**auditory fovea**' (Bruns (1976); Schuller and Pollak (1979); Neuweiler (1990); Kössl and Vater (1985); Kössl and Vater (1995); Vater and Kössl (2004)). In this region, the basilar membrane shows an overrepresentation in the frequency range of the CF's reference frequency, leading to a disproportionately large area, and therefore, to an enhanced resolution to discern frequencies around the reference frequency. Afferent projections lead to higher areas within the auditory pathway such as the **inferior colliculus (IC)** (Schuller and Pollak (1979)) and auditory cortex (Ostwald (1984); Suga *et al.* (1987)), which also present an overrepresentation of neurons in the range of the reference frequency (Schuller and Pollak (1979); Ostwald (1984)).

In these higher areas of the auditory pathway, the tonotopic organization reflects the cochlea's structure, with a pronounced concentration of yet another set of sharply tuned neurons in the range of the reference frequency, essential for processing the detailed flutter information contained in CF echoes (Pollak and Schuller (1981); Rübsamen *et al.* (1988)). For example, in the IC of *R. ferrumequinum*, approximately 50% of all neurons are tuned to these crucial frequencies, demonstrating a significant focus on the frequency range associated with flutter detection (Schuller and Pollak (1979)). This pattern continues in the auditory cortex, where neurons are specifically organized to prioritize and process frequencies that carry vital information about the motion and location of prey (Suga (1990); O'Neill (1995); Ostwald (1988)). These sophisticated adaptations of both transmitter and receiver systems allow CF bats to effectively decode the complex modulations within the echoes resulting from insect wing beats.

The modulations in echoes from fluttering insects present a distinctive prey cue, particularly in highly cluttered environments. The rhythmic pattern of amplitude fluctuations, coupled with frequency modulations due to micro-Doppler shifts from the moving insect wings, offers a unique acoustic signature that strongly contrasts with the non-modulating echoes from branches and leaves. Additionally, parameters such as the insect wingbeat frequency and wing size contribute to specific modulations in glint echoes. Behavioral studies have demonstrated that CF bats utilize the information contained in these acoustic

glints not only to detect but also to accurately characterize and classify their prey (Kober (1988); Kober and Schnitzler (1990); Von Der Emde and Menne (1989); Von Der Emde and Schnitzler (1990); Roverud *et al.* (1991); Koselj *et al.* (2011)).

1.5 Debate about insect flutter detection using FM echolocation

Thus far, we have seen how the intricate adaptations of both the transmitter and receiver systems in CF bats enable them to decode insect flutter information from complex echoes for effective foraging in cluttered environments. However, it remains a pertinent question whether bats that utilize **frequency-modulated (FM)** calls, which constitute around 80% of insectivorous bat species (Wilson and Mittermeier (2019)), are similarly capable of detecting and processing insect flutter information.

Studies ensonifying fluttering insects with short FM signals have shown that the resulting echoes exhibit modulations in amplitude and frequency due to the insects' wing beats (Roeder (1963); Kober and Schnitzler (1990); Moss and Zagaeski (1994)), indicating that flutter information should be accessible to FM bats. Psychophysical experiments in the laboratory further support this, demonstrating that trained FM bats can distinguish between rotating targets at different rates (Sum and Menne (1988); Roverud *et al.* (1991); Grossetête and Moss (1998)) and discern modulations in amplitude and delay that simulate flutter (Baier and Wiegrebe (2018); Baier *et al.* (2018)).

The debate surrounding the ability of FM bats to decode flutter information hinges on whether this decoding is achieved by analyzing the information contained in a single glint echo or in a series of successive echoes. Some studies propose that FM bats can discriminate between targets with different movement rates by analyzing the interference patterns created when echoes from a moving part, such as the rotating propeller, overlap with those from a stationary part of the apparatus (Sum and Menne (1988); Moss and Zagaeski (1994)). Specifically, when sound waves strike the propeller in a favorable position, they generate a Doppler shifted echo. This echo can interfere with the echo from the stationary part, producing a two-wavefront echo where the time gap between the two wave-fronts varies according to the wing's velocity (Sum and Menne (1988)). Similar two-wavefront echoes originate from the interference of the overlapping echoes from the moving wing of an insect and its "stationary" body (Moss and Zagaeski (1994)).

Other studies suggest that an insect's wing beat "is not encoded in a single echo but a sequence of echoes" (Kober and Schnitzler (1990)) and that FM bats might be able to estimate the flutter frequency of an insect by synchronizing their emission rate with the insect's wing beat (Moss and Zagaeski (1994)). Also, Fontaine and Peremans (2011) suggested that bats might use compressive sensing to reconstruct the wing beat of an insect from successive echoes even when undersampled (for more details, see Section 3.5.1).

However, in our opinion this debate lacks conclusive behavioral evidence under behaviorally relevant scenarios, that distinctly demonstrate FM bats use flutter information for effective prey detection, particularly in highly cluttered environments. We also consider that the echolocation behavior of the FM bat species used to resolve this issue so far may have been inadequate, since the call emission rates during the search phase of these species is relatively low to decode insect flutter information. Field recordings indicate that *Pipistrellus stenopterus* has an average **pulse interval (PI)** of 74.4 ms with an average **pulse duration (PD)** of 8.3 ms (Kingston *et al.* (2003)), and *Eptesicus fuscus* has PIs of 122 ms and PD of 11 ms (Surlykke and Moss (2000)). Such high PIs would correspond to a call emission rate of just 13.4 Hz in *P. stenopterus* and 8.2 Hz in *E. fuscus*. In contrast, species belonging to the so far understudied Natalidae family may be more suitable candidates to further study insect flutter detection using FM echolocation. With PIs during search phase ranging between 20-50 ms (Rydell *et al.* (2002); Sanchez *et al.* (2017); Murray *et al.* (2009)), these bats can achieve call emission rates between 20 and 50 Hz, suggesting that Natalids could be more effective in detecting and analyzing flutter information using FM echolocation.

1.6 The Natalidae family and *Natalus tumidirostris*

The Natalidae family, consisting of four genera—*Chilonatalus*, *Natalus*, *Nyctiellus*, and *Primonatalus*—and 13 extant species, is widely distributed across the Neotropics, including continental Central and northern South America and most Caribbean islands, with sporadic sightings in the Amazon rainforest (Tejedor (2011); Silva Taboada (1979)). Despite their broad geographic spread and ecological significance as dedicated insectivores and potential pest-controllers, the echolocation strategies and calls of these bats remain profoundly understudied. This gap in knowledge largely results from the chal-

lenges in field sampling, as Natalids rarely fall into harp traps or fly into mist nets, the primary methods for bat capture in the field.

We know from only limited studies in the field that Natalids often exhibit crepuscular activity and roost in caves, sometimes forming medium to large colonies with other species (Silva Taboada (1979)). They are characterized by their small size, with the smallest species, *Nyctiellus lepidus*, measuring just over 28.1 mm in forearm length and weighing as little as 2.3 g, and the largest, *Natalus primus*, reaching up to 51.2 mm and 12.6 g (Silva Taboada (1979); Tejedor (2011)). Adapted for maneuverability and slow flight, Natalids have low aspect ratio and very low wing loading, allowing them to navigate and forage near or between vegetation in narrow spaces, typically below 3.5 meters above ground (Miller (1904); Sanchez *et al.* (2017); Torres-Flores and López-Wilchis (2018)).

Very few studies have specifically investigated the echolocation behavior of Natalids, with most reports limited to call characteristics from single hand-releases. The calls from these bats are very faint, of very high-frequency (90-160 kHz), typically short (1.5-3.5 ms), downward frequency modulated, and with the energy predominantly concentrated in the second harmonic (Barataud *et al.* (2013); Murray *et al.* (2009); Rydell *et al.* (2002); Jennings *et al.* (2004); Sanchez *et al.* (2017)). As already mentioned, one of the main characteristic of interest to study Natalid species for insect flutter detection is their apparent high call emission rate due to the few reported sequences with pulse intervals ranging between 20 and 50 ms (Rydell *et al.* (2002); Sanchez *et al.* (2017); Murray *et al.* (2009)). However, Murray *et al.* (2009) only analyzed 28 calls of *Nyctiellus lepidus* and Rydell *et al.* (2002) only analyzed 5 sequences with a total of 52 calls of *Natalus stramineus*. The only thorough study on call characteristics of a Natalid species was done by Sanchez *et al.* (2017), where they analyzed 349 calls of *Natalus primus*. Unfortunately, since this study emphasized on characterizing the calls of this species for future conservation and monitoring studies, no data is provided regarding the pulse intervals or call emission rates. From Fig. 2D of that study, we can estimate that the PIs of *N. primus* range between 20 - 30 ms, corresponding to a 30 - 50 Hz call emission rate. As we can see, a thorough recording and analysis of the echolocation behavior of any Natalid species remains to be done. Similarly, a conclusive behavioral study focusing on insect flutter detection from species using FM echolocation with high emission rates is also yet to be carried out.

Since Natalids are very hard to capture using conventional field sampling methods, we chose to study the Trinidadian funnel-eared bat *Natalus tumidirostris*, because a well-established, accessible and healthy colony was reported in Santander, Colombia (Pérez-Torres *et al.* (2015)). This site provides a unique opportunity to capture and study these bats not only under semi-natural but also natural conditions and shed more light into its so far undescribed echolocation behavior and potentially reveal if and how FM bats can evaluate insect flutter information in echoes from highly cluttered environments.

1.7 Aims of this Thesis

This thesis sets out to achieve the following objectives:

- Record and thoroughly describe the echolocation behavior of the Natalid species *Natalus tumidirostris*, focusing to understand its foraging strategy.
- Compare our findings on the echolocation behavior of *N. tumidirostris* with the rest of the Natalidae family and determine if this species (and the Natalidae family) can be assigned to the Narrow Space Foragers bat guild.
- Explore *Natalus tumidirostris*'s preference for fluttering over non-fluttering prey and assess its reliance on flutter cues for detection.
- Analyze which specific flutter cues within the returning echoes are available for *N. tumidirostris* to detect prey in clutter-dense environments.
- Compare the echolocation behavior of *N. tumidirostris* with that of other FM bat species and discuss which echolocation strategies are most efficient for flutter detection in FM bats.
- Determine the dietary habits of *N. tumidirostris* to better understand its ecological role by assessing the arthropod community it forages on.
- Evaluate whether this bat species has a generalist or a specialist feeding strategy.
- Understand the connection between the foraging strategy of *N. tumidirostris* and the prey items it should be able to detect and capture.

Chapter 2

Echolocation behavior of *Natalus tumidirostris*: continuous sampling for detecting flutter motion in a FM bat?

2.1 Abstract

Echolocation systems and morphology of bats are adapted to where they forage, what they eat, how they find and how they acquire their food. While the foraging and echolocation behavior of a variety of bat species has been described in detail, members of the Natalidae family are highly understudied. In this study, we investigated the echolocation behavior of the natalid species *Natalus tumidirostris* while searching and hunting for prey inside a flight tent. During search *N. tumidirostris* emitted very faint, short, broadband, high frequency FM calls with the main energy in the second harmonic sweeping in the mean from 152.6 kHz to 105.1 kHz within 1.2 ms. The calls were emitted in a nearly continuous fashion with a mean pulse interval of 25.6 ms. Such continuous sampling while searching for prey has so far not been described for any other bat species. During approach pulse intervals were reduced to 6 ms before prey capture. In the inspection phase prior to the approach, pulse intervals were kept between 16 and 23 ms for several hundreds of ms. *N. tumidirostris* also approached a rotating blade simulating a fluttering insect. Morphological measurements revealed a small body size, very low wing loading and low aspect ratio, enabling slow and highly maneuverable flight. We conclude that morphology and echolocation behavior of *N. tumidirostris* and most likely of all Natalidae are especially adapted to search for and acquire prey with echolocation in highly

cluttered spaces. Continuous echolocation with broadband signals emitted at high repetition rates may be an adaptation to detect and evaluate flutter information in the echoes of insect prey and may enhance the discrimination of prey echoes from background echoes within densely cluttered environments.

2.2 Introduction

Ever since the research on bat echolocation began more than 80 years ago ([Pierce and Griffin \(1938\)](#), [Griffin \(1944\)](#), [Grinnell and Griffin \(1958\)](#)), a vast number of studies have revealed a phenomenal diversity of adaptations, strategies and behaviors echolocating bats have evolved to navigate and find food ([Fenton \(1990\)](#), [Fenton \(1995\)](#), [Schnitzler and Kalko \(1998\)](#), [Kunz and Fenton \(2003\)](#), [Schnitzler *et al.* \(2003\)](#)). All of the nearly 1200 species of the so far described echolocating bats ([Wilson and Mittermeier \(2019\)](#)) own a species-specific echolocation system to accomplish species-specific tasks in distinct environments. The main drivers in shaping not only these echolocation systems, but also flight morphologies are the habitats where bats forage, their diet type and how they find and acquire their food. Therefore, bats foraging under similar ecological conditions show similar adaptations in their motor and sensory systems ([Norberg and Rayner \(1987\)](#), [Schnitzler and Kalko \(2001\)](#), [Schnitzler *et al.* \(2003\)](#), [Denzinger and Schnitzler \(2013\)](#), [Denzinger *et al.* \(2016\)](#), [Denzinger *et al.* \(2018\)](#)). Likewise, morphology and echolocation system allow us to predict where and how a species likely forages for prey.

While the echolocation and foraging behaviour of a variety of bat species has been described, some remain to be studied and understood. The echolocation strategies and calls among the Natalidae family for example, a group comprised of 4 genera *Chilonatalus*, *Natalus*, *Nyctiellus* and *Primonatalus*) with 13 extant species ([Tejedor \(2011\)](#)), remain thoroughly understudied. This family of bats is distributed throughout the Neotropics, mainly in continental Central- and northern South America as well as in most insular systems of the Caribbean but has been also sporadically reported throughout the Amazon rainforest (see Fig. 2 in [Tejedor \(2011\)](#); [Silva Taboada \(1979\)](#)). All Natalids seem to have rather crepuscular activity and they roost in caves sometimes sharing roost with other species in medium/large size colonies ([Silva Taboada \(1979\)](#), [Tejedor \(2011\)](#), [Pérez-Torres *et al.* \(2015\)](#)). They have been described as small in size, with forearm lengths as low as 28.1 mm and a weight of 2.3 g for some of the smallest individuals of *Nyctiellus*

lepidus (Silva Taboada (1979)). The largest member of the family, *Natalus primus*, has a maximum forearm length of just 51.2 mm and a maximum weight of 12.6 g (Tejedor (2011)). All bats of the genus *Natalus* have wings with broad wingspan, large wing area and low wing loading, enabling them to fly slowly and with a high maneuverability (Torres-Flores and López-Wilchis (2018)). Field observations have reported for example that *Nyctiellus lepidus*, *Natalus stramineus* and *Natalus mexicanus* were seen foraging near or between vegetation, mostly below 3.5m above the ground and occasionally flying on ground level (Miller (1904), Sanchez *et al.* (2017), Torres-Flores and López-Wilchis (2018), López-Wilchis *et al.* (2020), López-Wilchis *et al.* (2021)). Their flight has been described as slow and butterfly-like, being able to hover between vegetation (Torres-Flores and López-Wilchis (2018)). Natalids seem to be strict insectivorous bats feeding on arthropods, mainly lepidoptera, as some few stomach content analysis have shown (Silva Taboada (1979), Tejedor *et al.* (2004), Santos-Moreno and Soriano-Cruz (2019)). A detailed fecal analysis of *Natalus mexicanus* revealed besides Coleoptera, Diptera and Lepidoptera a large amount of Araneae in the diet (Torres-Flores and López-Wilchis (2018)).

So far, it is unknown which echolocation strategy Natalid bats use to find and acquire their prey. Only a handful of research articles have described their echolocation calls, most likely because the calls of Natalids seem to be very faint and with a very high frequency. Echolocation calls described for *Natalus tumidirostris* (Barataud *et al.* (2013)), *Nyctiellus lepidus* (Murray *et al.* (2009)), *Natalus stramineus* (Rydell *et al.* (2002) & Jennings *et al.* (2004)) and *Natalus primus* (Sanchez *et al.* (2017)) share being short (1.5-3.5ms), downward frequency modulated (FM), high frequency (160-90 kHz) and multi-harmonic, with the energy mainly concentrated in the second harmonic. Most of these calls were recorded from hand release flights.

Only one study has analyzed the echolocation behavior of a Natalid species (*N. primus*) in some depth (Sanchez *et al.* (2017)). Here the researchers recorded the bats in a small cave gallery using a four-microphones-array (Sanchez *et al.* (2017)). However, the main focus of the research was to characterize the calls of this species for future conservation and monitoring studies and only describes the duration and frequency parameters in detail. Recordings from foraging or hunting behavior still remain unknown for this family. The objective of this research, in addition to expanding the database of Natalid calls, is thus to record and analyze the echolocation behavior of a Natalid species, particularly

while hunting. We chose the Trinidadian funnel-eared bat *Natalus tumidirostris* as our study object, because a well-established, accessible and healthy colony was reported in Santander, Colombia (Pérez-Torres *et al.* (2015)). We also expect this study to shed some light on the echolocation strategies of the Natalidae family.

2.3 Materials and Methods

We captured a total of 33 *Natalus tumidirostris* before dusk throughout 19 nights between the months of July and November 2018 inside the Macaregua cave (06°39'36.2" N, 073°06'32.3" W), located in Santander, Colombia. We also caught moths of the family Noctuidae using a custom-made LED insect light trap based on Gunnar Brehm's design (Brehm (2017)). The trap was set in a forest outside the cave from 17:30 to 20:00. We released each bat individually inside a flight tent, (L: 2.5 m; W: 2.5 m; H: 2.10 m) installed 1 km northwest from the cave. After approximately 15 min of acclimatization –the time span after which most bats usually started to search for prey– the living Noctuidae moths were released into the flight tent to trigger the bats' hunting behavior. Echolocation calls were recorded using four unidirectional custom-made ultrasonic microphones (Animal Physiology, University of Tübingen, flat frequency response within 6 dB from 20 kHz to 200 kHz). We mounted the microphones on four tripods arranged in a square in the middle of the flight tent. Each microphone was pointing upwards and had a lateral distance of 1 m from each other. All four channels were simultaneously recorded with the software AVISOFT RECORDER (AVISOFT, version 4.2.27), using an analog-digital converter (NI-USB 6356, National Instruments; sampling rate of 600 kHz for each channel). The settings in AVISOFT RECORDER were adjusted to trigger automatic recording for the following 5 seconds when a signal surpassed a 5 dB threshold within a 100 - 160 kHz bandwidth in any of the channels. Recordings had a minimum length of 5 s, but some of them were as long as 48 s when the bats were circling around the microphones. During the recordings, a researcher was taking notes at all times to assign the bat's behavior to the ongoing recording. We grouped the recordings as searching behavior, prey-approaching behavior or as a successful prey-catch event. When a bat was particularly active, we turned on an artificial wing beat motion device consisting of a battery-powered rotor drill (custom adapted PROXXON Micromot 60/e, PROXXON GmbH, Germany) to which we attached a small piece of tape strip with the

shape of a Noctuidae moth's wing. We calibrated the rotating frequency of the blade to approximately 25 Hz, which corresponded to the wing beat frequency of the Noctuidae moths the bats caught within the flight tent. This rotating blade was hand held by the researcher close to one of the microphones and left on for five minutes. The recording sessions took between 30 and 40 minutes per bat (including acclimatization). No more than 4 bats were recorded per night. After all bats were recorded, we brought them back to the entrance of the cave and released them.

2.3.1 Morphometric parameters

On the final four nights of the field work, a total of 16 bats were captured to record morphometric parameters exclusively. Each animal was weighed, and then Snout-Vent-Length (SVL) and forearm lengths were measured to the nearest of 0.1 cm. We finally took a picture of each individual with extended wings using a cellphone camera (OnePlus 6, Shenzhen OnePlus Science & Technology Co. Ltd., China). After this, the animals were immediately released at the entrance of the cave. Later, using the software ImageJ (version 1.52p, Wayne Rasband, National Institutes of Health, USA), we analyzed each picture to estimate both wing span (B) and wing membrane area (S) (including the uropatagium) according to the methods described in [Norberg and Rayner \(1987\)](#). The measurements of SVL and forearm served to calibrate each image. Finally, we computed the values for aspect ratio (AR) and wing loading (WL) as follows: $AR = B^2/S$ and $WL = m * g/S$; with m as the mass of the bat in kg and g as the gravitational acceleration constant ($9.81 m/s^2$).

2.3.2 Data Analysis

We separated a total of 30 audio recordings from 13 individuals with a comparably good signal-to-noise ratio where *N. tumidirostris* bats were searching for prey. The recordings were then analyzed with a custom-made software written in Python (BAT-Spy, <https://github.com/jfsehuanes/BATSpy>).

Call time detection and extraction

To extract the precise times of bat calls from our 4-channel audio recordings, we transformed the audio into spectrograms (FFT=512; overlap=0.7, interpolation='hanning').

A dynamic threshold was calculated by computing the minimum of the average power within a 50-180 kHz bandwidth across the frequency axis for each channel. We marked every local peak after that threshold-crossing as a potential bat call. We then used a sliding window mechanism (window length = 50 ms) across the time dimension of the spectrogram to compare the average power of these detected peaks within each window across all channels. The channel that exhibited the highest average power for potential calls in a given window was deemed to have the best recording of the bat calls for that specific time-window. To finalize the extraction, the times of calls were manually overlooked to eliminate duplications and closely-spaced detections.

Parameter Extraction

Once the call times within a recording were clean, our next step was to extract the call parameters of the second harmonic of each call:

1. **Channel Selection.** The first objective was to determine the most optimal channel, i.e., the channel at which the call was loudest. For each call, the best channel was defined as the one that manifested the highest energy within the frequency range of 50-180kHz.
2. **Spectrogram Generation.** Using the selected channel, we generated a 5 ms short spectrogram (FFT=128; overlap=0.6, interpolation='hanning'). We centered this spectrogram around the time of the energy peak of the call (0dB).
3. **Noise Level Verification.** Before extracting the parameters of a call, we ensured the mean noise (defined as the average energy of the short spectrogram within a 250-300kHz bandwidth) was at least 7dB below the -12dB detection threshold set for crossings. Calls that didn't meet this criterion were skipped. Note that the calls produced by *N. tumidirostris* were very faint and so we had a considerable amount of calls from which we couldn't extract the call parameters.
4. **Curve Generation.** To extract relevant parameters, we generated two curves: The first curve was derived from the maximum power throughout the time axis of the spectrogram. The second curve was formulated by charting the maximum power alongside the frequency axis.

5. **Crossing Detection.** Utilizing a threshold crossing algorithm, we detected crossings in the curves with both positive and negative slopes. The set threshold for this detection was -12dB relative to the energy peak of the call. The crossing characterized by a positive slope indicated the start, while the crossing with a negative slope signified the end. By applying this logic to both the time and frequency curves, we successfully determined the start and end frequencies as well as the start and end times of each call. Lastly, we calculated the duration of the call by subtracting its detected start time from its end time. Though this semi-automated method for parameter extraction was the best result after thorough optimization, we believe the real call durations and bandwidths of *N. tumidirostris* to be slightly larger than what we registered. We therefore used exclusively pulse intervals when doing comparative analysis between different behaviors. On top of that, we computed the intervals using the difference in time between succeeding peak frequencies, instead of the commonly used start frequencies, because we realized this gave a more robust value given the limitations of our recording system, as well as our call-parameter-extraction algorithm.

All further analysis and figures were produced using custom written scripts in Python3.9.

2.4 Results

We investigated the echolocation behavior of *Natalus tumidirostris* while hunting in controlled semi-natural conditions in a flight tent. The bats were highly maneuverable and showed no difficulties to fly within this confined space. (see supplemental video). We observed them flying between the poles of our tripods, being able to perform tight turns in small spaces. The flight of *N. tumidirostris* looked characteristically “butterfly-like” and slow. Morphometric measurements revealed an average snout-vent-length (SVL) of 45.0 mm (± 1.4 mm, $n=16$) and a forearm length of 38.2 mm (± 0.9 mm, $n=16$). Wing- and tail membranes were conspicuously large, from which we estimated an average wing loading of 5.118 Nm^{-2} (SD $\pm 1.038 \text{ Nm}^{-2}$; $n=16$) and an aspect ratio of 6.134 (SD ± 0.637 ; $n=16$). A summary of the morphometric parameters is shown under Table 2.1.

Table 2.1: **Morphometric parameters of *Natalus tumidirostris***. For each parameter the mean value $\pm$ std is shown. Abbreviations: **SVL**:Snout-Vent-Length; **FA**: forearm. n=16

SVL (mm)	FA length (mm)	weight (g)	wingspan (m)	wing area (m ²)	aspect ratio	wing loading (Nm ⁻²)
45.0 $\pm$ 1.4	38.2 $\pm$ 0.9	5.6 $\pm$ 0.4	0.258 $\pm$ 0.012	0.011 $\pm$ 0.002	6.134 $\pm$ 0.637	5.118 $\pm$ 1.038

The echolocation calls of these bats were extremely faint: our microphones were unable to register a call if a bat was beyond 1-1,5m distance. A typical echolocation call of *N. tumidirostris* consisted of a downward frequency modulated (FM) sweep, with the main energy of the call focused on the second harmonic (see Fig. 2.1). During search flight, these bats produced high frequency and broadband FM calls with mean call durations of 1.2 ms (SD $\pm$ 0.4 ms; min=0.5 ms; max=2.9 ms; n=4508) and an average pulse interval of 25.6ms (SD $\pm$ 5.9 ms; n=4612; (see Figs. 2.2)). The calls had an average start frequency of 152.6 kHz (SD $\pm$ 12.5 kHz, min=113.2 kHz; max=185.2 kHz; n=4508), a peak frequency of 124.6 kHz (SD $\pm$ 11.2 kHz; min=98.8 kHz; max=170.8 kHz; n=4508) and an end frequency of 105.1 kHz (SD $\pm$ 7.5; min=74.8 kHz; max=137.2 kHz; n=4508) (see Fig 2.2D). From these values, we calculated an average bandwidth of 48.5 kHz for the second harmonic. The average duty cycle was 4.7% (SD $\pm$ 1.7; n=4102) (see Fig. 2.2C). Due to the faintness of the calls and the -12 dB criterion we used for parameter extraction the mean values are most likely underestimated. Signals with good signal to noise ratio such as the search signal shown in Fig. 2.1 had durations around 2ms and a bandwidth of around 80 kHz with start frequencies around 163 kHz and end frequencies around 83 kHz.

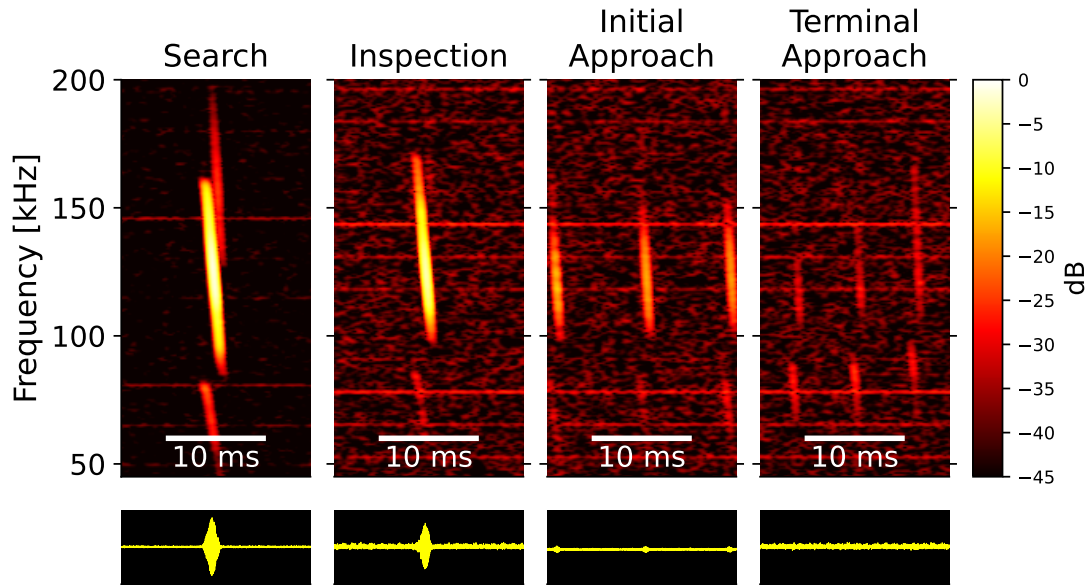


Figure 2.1: **Call repertoire of *Natalus tumidirostris***. Each panel shows a spectrogram of a typical call during search, inspection, the beginning and the end of the approach with its corresponding oscillogram on the bottom. Note the energy of the call is focused on the 2nd harmonic during search, inspection and the beginning of the approach, but is then shared between 1st and 2nd during the terminal part of the approach.

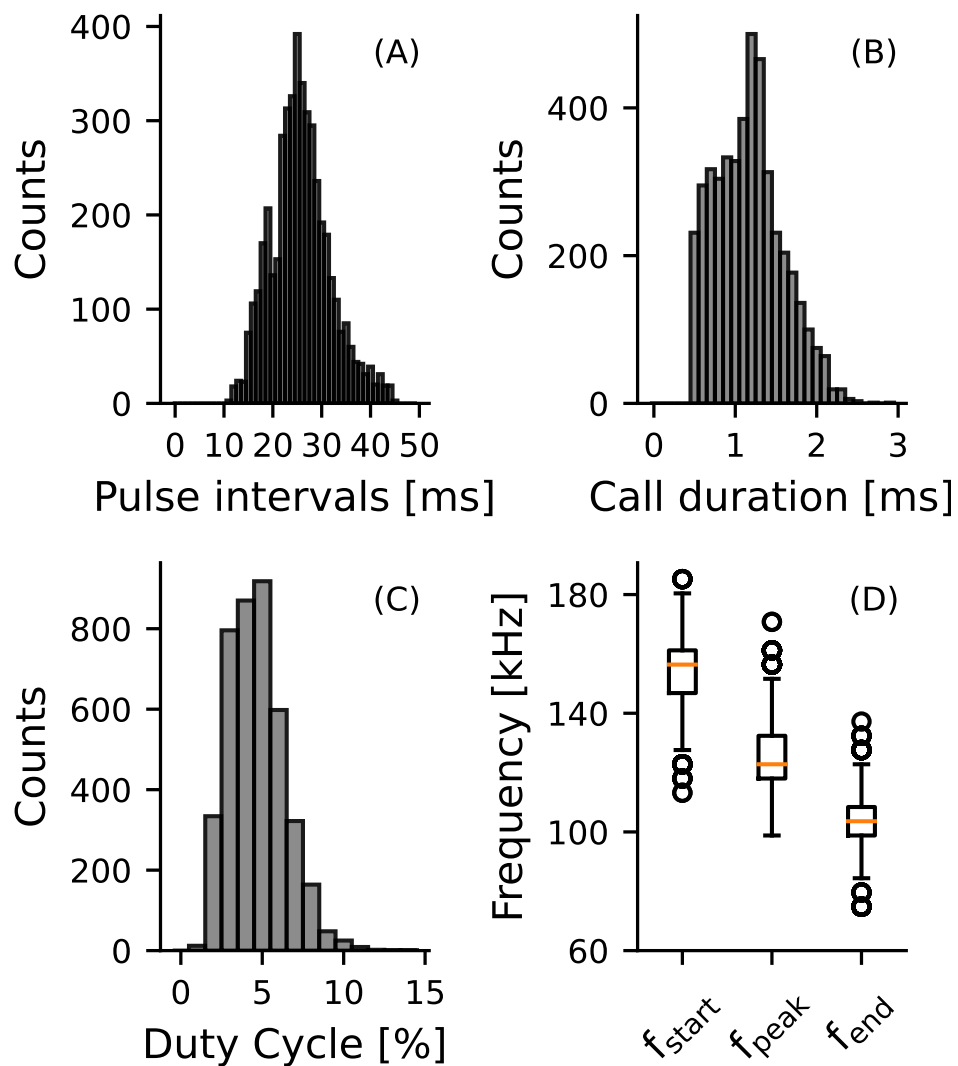


Figure 2.2: **Distributions of call parameters during the search phase.** (A) shows the distribution of the pulse intervals, while (B) shows the distribution of call durations. (C) shows the computed duty cycle and (D) shows three boxplots illustrating the distributions of start-, peak- and end-frequency of the calls.

While searching for flying moths, the calls were emitted in a nearly continuous fashion. We were able to register this continuous emission of calls without grouping or longer pulse intervals over a period of up to 2.5s (Fig. 2.3). We observed this continuous echolocation behavior throughout all recordings.

Considering that due to the low signal-to-noise ratio, start- and end frequencies as well as call durations were unreliable to extract from faint calls (see Section 2.3.2), we evaluated most of the bat's behavior by analyzing how the pulse intervals changed over time. During the search phase, we observed a slightly jittering pulse interval that ranged mostly between 20 and 35 ms (see Fig. 2.4A). When catching prey, we observed a clear reduction of both pulse intervals and jitter prior to the approach (see Fig. 2.4B). We denominated this phase as inspection because the calls were emitted shortly before the approach when the bat was already in the vicinity of the prey. During this inspection, which in the sequence shown under Fig. 2.4C lasted nearly 700 ms, the pulse intervals spanned between 16 and 23 ms. The subsequent approach was characterized by a continuous reduction of the pulse intervals all the way down to 6ms right before the prey capture. From the calls during inspection and the few approach calls with good signal to noise ratio we concluded that frequency parameters did not change substantially compared to search flight (see Fig. 2.1). It is also worth noting a relative increase in energy of the first harmonic during the terminal part of the approach (compare the relative difference in energy of 1st and 2nd harmonics across the different calls of Fig. 2.1).

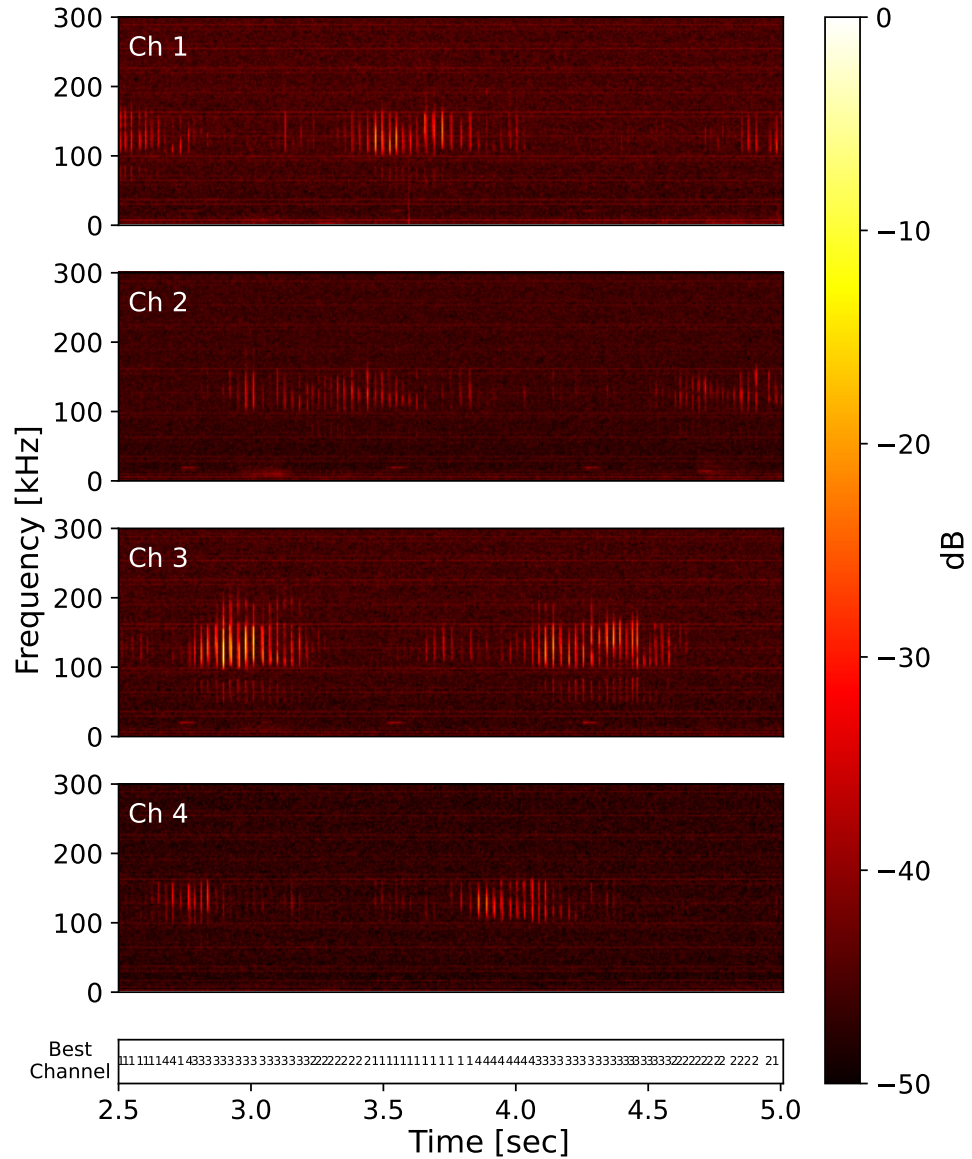


Figure 2.3: **Continuous echolocation during search.** This figure shows four spectrograms, corresponding to the four channels of a single example recording of *N. tumidirostris* during search. The panel on the bottom combines the calls detected from all channels and shows each call as a number indicating the channel where it was loudest (for details see Section 2.3.2). Note that during the 2.5s length of this example recording, the bat produced calls continuously.

When we presented a rotating blade simulating a fluttering insect, bats approached the device without making contact. However, in the echolocation behavior these bats reacted to a rotating blade (see Section 2.3) with a similar call pattern to when they approached one of the moths: they also lowered the pulse intervals and the jitter as they did during the inspection phase (compare Fig. 2.4D and C). However, after "inspecting" the rotating blade, the bats slowly increased the pulse intervals back to the standard search values, instead of proceeding with the approach. This behavior was registered in more than one opportunity and also with different individuals.

On the night of November 12th 2018 we observed some free flying *N. tumidirostris* bats came to visit the recording site between 22:00 and 23:00 hrs. The visiting bats only circled around the flight tent for a couple of minutes before leaving again. The visit of these conspecifics sabotaged the behavioral observation of the focal animal flying inside the tent, for it instantly became distracted by the visitors. However, we found this a very interesting event and recorded the echolocation calls of the visitors. An example sequence of such a recording is shown in Fig. 2.4B. Under free flying conditions *N. tumidirostris* also continuously produced calls with pulse intervals ranging between 20 and 40ms, supporting our observation that this is the general echolocation behavior of this species.

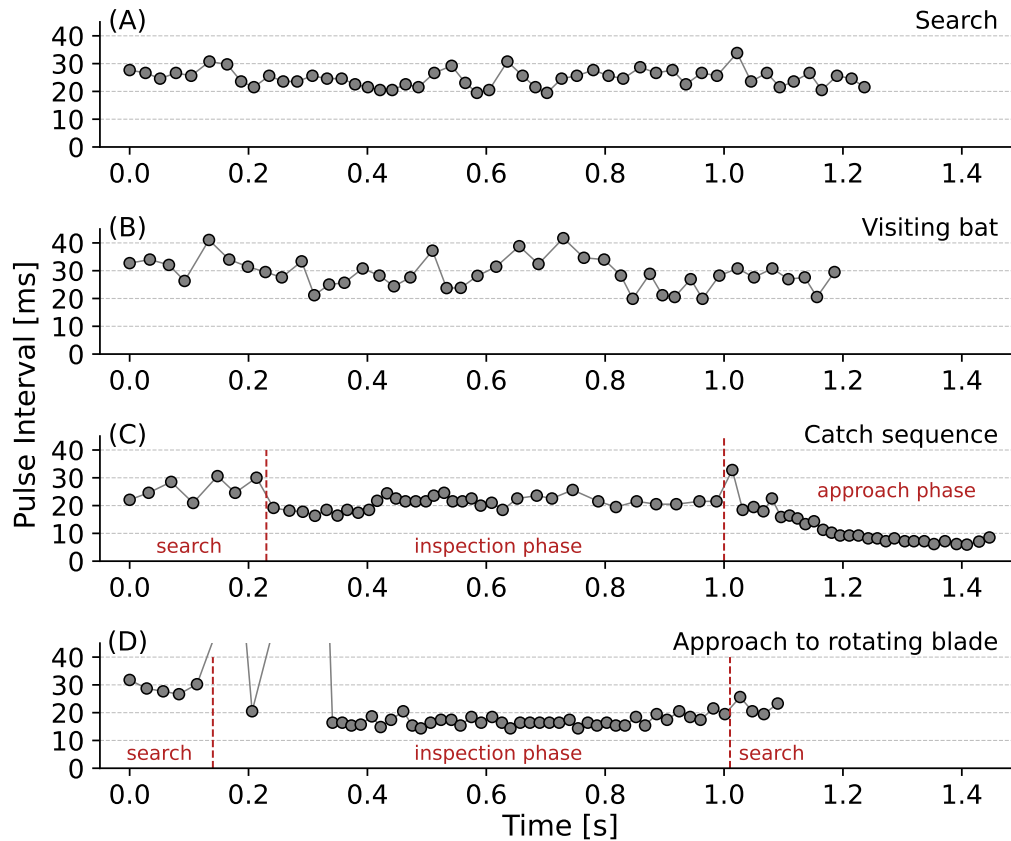


Figure 2.4: **Pulse intervals at different behaviors.** Each subplot shows the time of a call plotted against the time interval of a succeeding call. Note that in (A) and (B) all pulse intervals jitter above 20ms, corresponding thus to search behavior. In (C) we can observe a complete catch sequence. At the beginning, the bat searched with pulse intervals jittering above 20ms, following an inspection period where the values descended and stabilized around 20ms. Then came the approach in which the pulse intervals successingly descended down to 6ms in the terminal phase. (D) shows an example sequence when a bat got interested in a rotating blade. Unfortunately, our recording system didn't register loud enough calls between second 0.14 and second 0.32, hence the missing calls. Note however, that during the inspection part, the pulse intervals here resembled those during the inspection part of the catch sequence in (C). In this case however, the pulse intervals returned to standard search values after the inspection part, instead of continuing with the approach phase.

2.5 Discussion

In this research we described how the funnel-eared-bat *Natalus tumidirostris* searches for prey using 1 to 2 ms short, broadband calls with very high frequency. The most interesting observation was that the calls are produced continuously without grouping with a mean interval of 25 ms while searching for prey (see Fig. 2.3). This echolocation behavior was to our knowledge so far undescribed, and our results evidence this species has indeed a very interesting strategy for hunting prey. In contrast, bats that are specialized for hunting in narrow spaces and utilize frequency modulated echolocation signals tend to organize their calls in groups, typically producing two to three calls with each wingbeat, as documented in various studies (Thies *et al.* (1998), Schnitzler *et al.* (2003), Denzinger and Schnitzler (2013)). Yet it is very rare for narrow space hunters to reduce their pulse intervals below 40 ms during the search phase (see Siemers and Schnitzler (2004)). To our knowledge, the continuous echolocation strategy of *N. tumidirostris* with a repetition rate of 40 Hz during search has not been described for any other bat species.

One could argue the continuous echolocation behavior of *N. tumidirostris* is not a standard for searching prey, but rather a consequence of the confined space in the flight tent these bats were flying (L:2.5 m; W:2.5 m; H:2.10 m). We believe however this was not the case for two reasons: first, the bats were flying and maneuvering effortlessly inside the tent (see supplemental video), even in the confined space between the microphone tripod legs. Second, the interval between pulses was similar in recordings we took from free flying *N. tumidirostris* (see Fig. 2.4B) that spontaneously visited the recording site on one night, probably attracted by the sound of conspecific calls. We are convinced *N. tumidirostris* sustains continuous echolocation during search not only for a few seconds, but that it's rather the standard echolocation behavior for this species. However, our recording setup consisting of only four directional microphones combined with the faintness and the high directionality of the high frequency calls produced by this species made it impossible to register a sequence of calls for longer periods of time.

The echolocation behavior of other members of the genus *Natalus*, and overall the family *Natalidae* is poorly investigated, but from the few information on pulse intervals, duration and frequency parameters from other species, we conclude that this continuous echolocation behavior with short high frequency broadband calls is a shared trait within members of the *Natalidae* family. For example, *Nyctiellus lepidus* has pulse intervals of

31.9 ± 1.3 ms, a call duration of 2.7 ± 0.1 ms in average and a call bandwidth of 42.7 ± 1.2 kHz that ranges between 70.9 ± 0.4 kHz and 113.6 ± 1.0 kHz (Murray *et al.* (2009)). Other examples include *Natalus stramineus* with pulse intervals ranging between 24 and 55 ms, a call duration of 2.0 ± 0.3 ms and a frequency bandwidth of 50 kHz that ranges between 100-150 kHz (Rydell *et al.* (2002) & Jennings *et al.* (2004)); *Natalus primus* has a call duration of 1.7 ± 0.5 ms and a frequency bandwidth of 50.3 ± 9.8 kHz, ranging between 64.0 ± 8.0 kHz and 114.4 ± 5.5 kHz (Sanchez *et al.* (2017)).

In the study by Sanchez *et al.* (2017), no explicit values on the pulse intervals are reported, but we estimated these to lay between 20 and 40 ms, according to what they show in Figure 2D. The only study to our knowledge that shows call parameters for *N. tumidirostris* is by Barataud and collaborators (Barataud *et al.* (2013)), where from 5 recorded calls during a single hand release they report an average call duration of 3.5 ms (± 0.1 ms) and a frequency bandwidth of 77.4 kHz (± 29.5 kHz), but no values on pulse intervals are mentioned. The call characteristics we describe in this study (Fig. 2.2) are in line with what was published by Barataud and collaborators, however, we suspect the up to 2 ms difference in call duration recorded in that study is a product of the hand-release recording method, as well as the constraints of our method to extract call parameters.

Though we were unable to find and record where *N. tumidirostris* bats hunted naturally, we predict this species is suited for hunting in narrow spaces like a cluttered forest understory. We have come to this prediction for three main reasons. First is the morphology of this species. Weighing less than 6 g on average while having a large wing area leads to one of the lowest values for wing loading reported for bat species (compare Table 2.1 of this study with Table 1 in Norberg and Rayner (1987)). These features, added to an also low aspect ratio, gives *N. tumidirostris* the flight maneuverability required for hunting and avoiding obstacles in narrow spaces. Species with similar low wing loading and average aspect ratios tend to belong to the narrow space foragers guilds (Jennings *et al.* (2004), Senawi and Kingston (2019), Norberg and Rayner (1987), Norberg and Norberg (2012)). Second, observations from other researchers on other Natalid members also support the idea that members of this family hunt in narrow spaces. Miller reported *Nyctiellus lepidus* "flies very low, about bushes, and close to buildings" (Miller (1904)), while *Natalus stramineus* "can fly very slowly in clutter and hunts by slow hawking and/or by gleaning" (Jennings *et al.* (2004)). Additionally, we observed in this study that *N. tumidirostris* was able to hover right above the ground level, as well as nav-

igate through narrow spaces e.g., the gaps between microphone tripod legs effortlessly. Third is the characteristics of their echolocation calls. The short, very faint, broadband calls of very high frequency of *N. tumidirostris*, which are emitted with rather high repetition rate are especially suited to forage in dense vegetation. Short signal durations of 1-2 ms keep the signal and clutter overlap zone small, which reduces forward and backward masking, and allows bats to forage close to background (Schnitzler and Kalko (1998), Schnitzler and Kalko (2001)). High frequencies and low call amplitudes are adaptations to reject clutter and reduce masking. High frequencies are strongly affected by atmospheric attenuation (Lawrence and Simmons (1982), Yovel *et al.* (2011), Stilz and Schnitzler (2012)). Very low source levels combined with high frequencies reduce the range, the signals travel and minimize clutter echoes from on axis vegetation (Jakobsen *et al.* (2013)). Additionally, high frequencies have a high directionality resulting in a narrow sonar beam, which also reduces off axis clutter echoes (Surlykke *et al.* (2009), Jakobsen and Surlykke (2010), Jakobsen *et al.* (2013)). Moreover, broadband FM signals are suited for precise localization and deliver spectral cues for prey classification (Schnitzler and Kalko (2001)). Behavioral studies revealed that bandwidth highly correlates with the sensory access to prey close to vegetation (Siemers and Schnitzler (2004), Schmieder *et al.* (2012)). Furthermore, pulse intervals of about 25 ms in *N. tumidirostris* ensure a high update rate to avoid collisions in cluttered environments.

All bats that forage in narrow space have to solve a pattern recognition task to find prey. Narrow space bats that detect prey with echolocation have to distinguish between an echo train of background echoes alone and an echo train that additionally includes an echo from prey (Denzinger *et al.* (2018)). Detection and classification of prey echoes is facilitated if echoes from prey contain prey specific signatures. A possible strategy to distinguish between prey and background echoes is to evaluate flutter information in the echoes from insects, as has been shown for narrow space flutter detecting foragers (for review see Schnitzler and Denzinger (2011)). These bats emit calls with a long constant frequency (CF) component at high duty cycle. Echoes from fluttering insects are characterized by amplitude and frequency modulations in the CF part, so called glints, which are generated in the rhythm of the insect's wing beat (e.g. Schnitzler and Flieger (1983), Kober and Schnitzler (1990)). The glint pattern encodes prey specific information on wing beat rate, size and aspect angle which facilitates the classification of prey (Von Der Emde and Menne (1989), Von Der Emde and Schnitzler (1990), Roverud *et al.*

(1991), [Koselj et al. \(2011\)](#)). Long constant frequency calls, Doppler shift compensation and a highly specialized hearing system with an auditory fovea are adaptations for the detection and evaluation of flutter information in narrow space flutter detecting foragers ([Schnitzler and Denzinger \(2011\)](#))

Whether or not bats that emit frequency modulated calls are also able to perceive flutter information in echoes has been the subject of discussion in several studies. Ensonification of fluttering insects with short FM signals at high repetition rates revealed that insect wing beats indeed produce distinct changes in amplitude and in the dominant frequency band of consecutive returning echoes ([Kober and Schnitzler \(1990\)](#), [Moss and Zagaeski \(1994\)](#)). Amplitudes were highest if the glint coincided with the duration of the FM signal ([Kober and Schnitzler \(1990\)](#)). The frequency of high amplitudes strongly depends on the relative timing between the wing beat motion and the impinging sound as well as the angle at which a fluttering insect wing is hit by the impinging soundwaves ([Moss and Zagaeski \(1994\)](#)). The resulting pattern in sequential echoes with alternating differences in amplitude and spectral parameters may convey information about the wingbeat of prey even when undersampled ([Fontaine and Peremans \(2011\)](#)). Also, behavioral studies have revealed that trained FM bats can discriminate between two rotating targets at different rates ([Sum and Menne \(1988\)](#), [Roverud et al. \(1991\)](#), [Grossetête and Moss \(1998\)](#)). In these experiments, the sampling rate these bats used was rather low, suggesting that flutter information cannot be conveyed in the sequence of echoes but must be encoded in single echoes. [Sum and Menne \(1988\)](#), as well as [Grossetête and Moss \(1998\)](#) suggested that bats use the interference pattern generated by Doppler induced changes in echo delays to discriminate between different rotation rates. When the impinging sound hits the rotating blade in the favorable position, the moving part of the wing beat simulator generates a Doppler shifted echo which interferes with the echo of the stationary part of the apparatus, creating a two-wavefront echo, where the time separation between the two echoes varies with the velocity of the moving rotor blade. Such interference patterns are also created by overlapping echoes from an insect's body and its moving wings and could convey sufficient information that the echo comes from a fluttering insect ([Grossetête and Moss \(1998\)](#)).

In our opinion, behavioral evidence is still missing to demonstrate whether FM bats indeed make use of flutter information to detect and classify prey items under natural conditions. Although our current results on the echolocation behavior of *Natalus tu-*

midirostris do not prove whether this species is able to perceive flutter motion from prey items, we suggest it is a good candidate to further study this matter. Strong evidence for the evaluation of flutter information in this species comes from the observation that several individuals approached a rotating blade we presented them while they were hunting thereby showing a similar echolocation behavior as when they approached a real moth (compare Fig. 2.4C with D). The almost continuous echolocation with pulse intervals around 25 ms in search and below 20 ms during the rather long inspection may generate a specific pattern in amplitude and spectral composition of consecutive echoes and/or deliver a sufficient number of echoes with glints for the evaluation of flutter information encoded in a single echo. Continuous echolocation in *N. tumidirostris* and most likely in all other Natalids may thus be an adaptation to detect and evaluate flutter information in echoes from FM calls and may improve the discrimination of prey echoes from background echoes within densely cluttered environments.

Chapter 3

Preference for fluttering targets reveals the use of flutter information in the FM bat *Natalus tumidirostris*

3.1 Abstract

Bats that forage for insects in narrow spaces like dense vegetation face the problem that signal echoes from prey items are often masked by much louder echoes from background. In a previous study that characterized the echolocation behavior of *Natalus tumidirostris* (see Chapter 2), we hypothesized that the continuously produced frequency modulated (FM) echolocation calls with short call intervals while searching for prey could be a suitable echolocation strategy to extract flutter information from insects hidden in cluttered spaces. In this study, we used both behavioral experiments in a semi-natural environment, as well as Monte Carlo simulations to investigate this hypothesis.

We captured a total of 39 bats and released them individually in a flight tent where we presented the bats with target moths that varied in motion and flutter to discern their prey selection preferences. This was complemented by recording and analyzing the bats' echolocation calls across the different hunting phases—search, inspection, and terminal approach—to better understand how this species is detecting and discriminating its prey.

Our results show that *N. tumidirostris* exhibits a strong preference for fluttering, over non-fluttering moths, with a significant statistical deviation from chance. This result confirmed *N. tumidirostris*' ability to perceive and react to flutter cues from prey items. Furthermore, we demonstrated that *N. tumidirostris* effectively locates and captures prey hid-

den in dense vegetation within our semi-natural flight tent setup, suggesting its echolocation strategy is well suited for hunting in such environments.

Our acoustic analysis revealed distinct patterns in echolocation call parameters across the different hunting phases. Most importantly, we observed that *N. tumidirostris* used shorter pulse intervals during the on average 0.6 s long inspection phase, and we discuss how this behavior can be a strategic adjustment not only to detect flutter, but also to estimate the flutter frequency of an insect. Also, our Monte Carlo simulations demonstrated how many acoustic glints (a key signal in echoes from fluttering insects) can be detected by FM bats, given a combination of their call duration and pulse intervals. With this, we observed that the continuous echolocation behavior of *N. tumidirostris* enhances its ability to detect such acoustic glints. Finally, we discuss and challenge previous assumptions that only bats producing constant frequency (CF) calls are capable of flutter detection, highlighting the adaptability and complexity of FM echolocation strategies.

3.2 Introduction

Hunters use a variety of signals and strategies to detect and characterize their prey. Many insectivorous bats can detect and locate their prey by means of echolocation. Yet the detection of relatively faint echoes of insect prey can be very challenging when they overlap with loud echoes from leaves and branches in cluttered spaces.

Bats that hunt within this so-called narrow space, where prey echoes are masked by background echoes, have evolved unique echolocation and foraging strategies to locate their prey (Schnitzler *et al.* (2003); Denzinger and Schnitzler (2013); Surlykke *et al.* (2016); Denzinger *et al.* (2018); Schnitzler and Denzinger (2024)). For instance, narrow space passive gleaning foragers utilize cues produced by prey for detection and location, and employ echolocation primarily for navigating in space and avoiding obstacles (Bell (1982); Bell (1985); Arlettaz *et al.* (2001)). Narrow space active gleaning foragers analyze the spectral and temporal patterns within the complex echo trains of their calls, which include the echo from the prey, and those from surrounding clutter. To uncover insects on foliage, they deploy very short, high-frequency FM signals and strategically orient themselves to optimize the timing between clutter and prey echoes. This careful positioning enables them to differentiate between leaves with and without resting prey (Denzinger and Schnitzler (2004); Geipel *et al.* (2013); Geipel *et al.*

(2019); Clare and Holderied (2015)). Narrow space flutter-detecting foragers specialize in discerning amplitude and frequency modulations—termed “acoustic glints”—in the echoes of their elongated, **constant frequency (CF)** calls. These modulations occur as sound waves meet the insect wings perpendicularly, mirroring the wings’ beating rhythm (Roeder (1963); Schnitzler and Flieger (1983); Kober (1988); Kober and Schnitzler (1990)). Adaptations such as Doppler shift compensation and a highly specialized hearing system equipped with an auditory fovea are crucial for these bats to identify and assess flutter movements in cluttered environments (Schnitzler and Denzinger (2011)).

So far, flutter detection and evaluation has only been proven for bats producing CF echolocation calls (Von Der Emde and Menne (1989); Von Der Emde and Schnitzler (1990)). However, around 80% of insectivorous bats (Wilson and Mittermeier (2019)) produce calls that are **frequency modulated (FM)**. Whether or not FM bats can also perceive flutter information has been a subject of discussion in several studies. Psychophysical experiments in the laboratory with rotating propellers have shown that FM bats can discriminate between targets with different flutter frequencies (Sum and Menne (1988); Roverud *et al.* (1991); Grossetête and Moss (1998)) or between amplitude or delay modulations that simulate flutter (Baier and Wiegrebe (2018); Baier *et al.* (2018)). Also, Fontaine and Peremans (2011) discussed that bats with short FM signals might be able to reconstruct the wing beat rate of an insect by using **compressive sensing (CS)** and Moss and Zagaeski (1994) speculated that bats might synchronize their emission rate to the wing beat rate of the insect to determine the insect’s flutter frequency. Yet in our opinion, behavioral evidence that clearly demonstrates the use of flutter information to detect and classify prey items in cluttered spaces by FM bats is still missing (for details, see Section 2.5).

In a previous study, we described the echolocation behavior of the FM bat *Natalus tumidirostris* (see Chapter 2). This species found in the neotropics forages in cluttered spaces and produces 1.2 ms short, high frequency FM echolocation calls with a start frequency around 153 kHz and a large bandwidth of around 80 kHz. More importantly, *N. tumidirostris* produces its calls almost continuously while searching for prey with a mean **pulse interval (PI)** of $25.6 \text{ ms} \pm 5.9 \text{ ms}$. With such a high sampling rate of the environment, we assumed that this bat species may use flutter information in the echoes of its FM echolocation calls to detect and possibly also to classify fluttering prey in cluttered spaces. Furthermore, we found strong evidence for the evaluation of flutter

information in this species from observing several individuals reacting and approaching a rotating blade we presented them while they were hunting, thereby showing a similar echolocation behavior as when they approached a real moth (compare Fig. 2.4 C with D).

With this research we aimed to: (i) explore *Natalus tumidirostris*'s preference for fluttering over non-fluttering prey and assess its reliance on flutter cues for detection; (ii) analyze which specific flutter cues within the returning echoes are available for *N. tumidirostris* to detect and possibly evaluate prey in clutter-dense environments; and (iii) compare the echolocation behavior of *N. tumidirostris* with that of other FM bat species and discuss which echolocation strategies are most efficient for flutter detection in FM bats.

3.3 Materials and Methods

We captured a total of 39 *Natalus tumidirostris* bats before dusk during the nights of November 18th - 22nd 2019; December 4th - 7th 2019 and February 11th - 15th 2020 at Macaregua cave (06°39'36.2" N, 073°06'32.3" W), located in Santander, Colombia. The Noctuidae moths we used as targets for the experiment were caught with a custom-made LED insect light trap based on Gunnar Brehm's design (Brehm (2017)). The trap was set in the forest outside the cave from 17:30 to 20:00.

We released each bat individually inside a flight tent, (L:2.5 m; W:2.5 m; H:2.10 m) installed 1 km northwest from the cave. After nearly 20 min of acclimatization –when bats usually started to search for prey– the targets were hung as shown in Figure 3.1 for 25 min. Each target consisted of a wire-tethered moth, hanging 50 cm from the cage's ceiling. We placed the targets in the center of the cage, with a 120 cm lateral distance from one another. The targets were as follows: i) a fluttering-moving target tethered to a 0.4 mm $\varnothing$ wire, ii) a fluttering, but immobile target due to a thicker 1.0 mm $\varnothing$ wire, iii) a non-fluttering but movable target via a pulley system and iv) a non-fluttering and immobile target. We sacrificed the moths of the non-fluttering targets right before the beginning of the experiment. Also, as soon as there was an approach with catch to any of the targets, we replaced it right away with a new one.

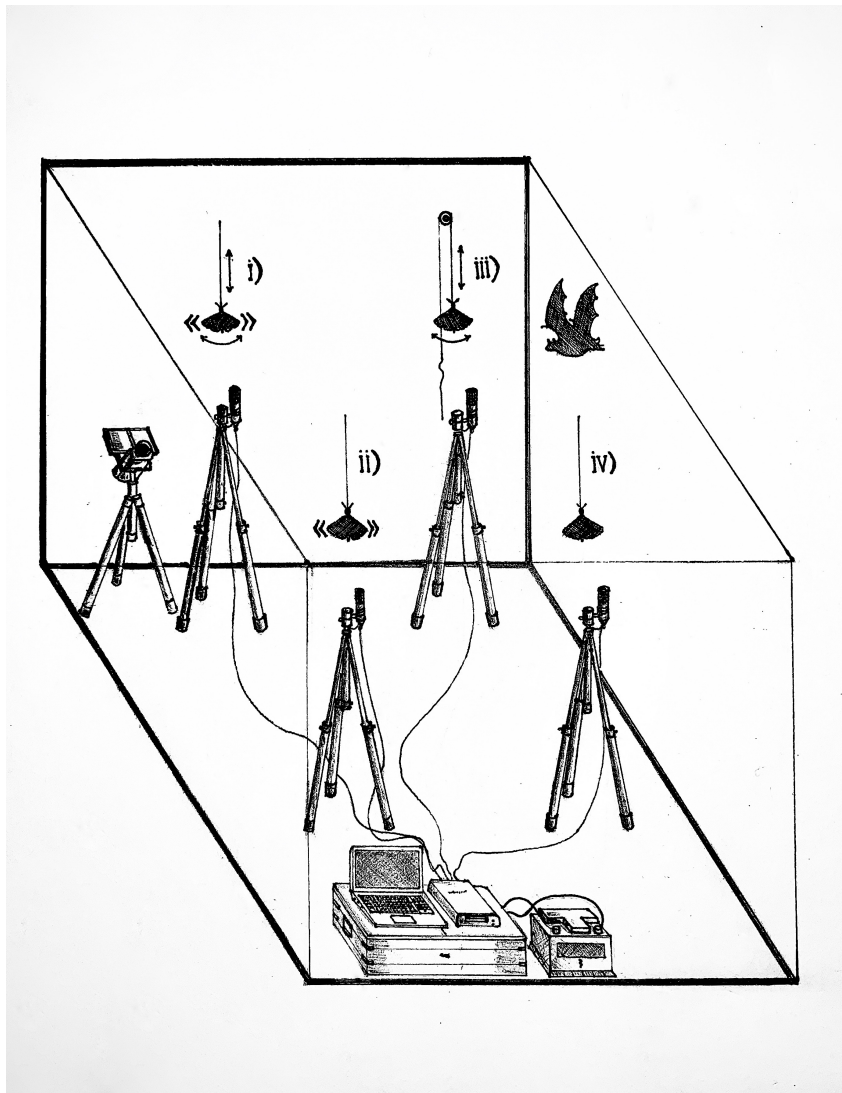


Figure 3.1: **Experimental set-up sketch of the flutter-preference experiment.** Each bat had four moth targets to choose from: i) a fluttering-moving moth tethered to a 0.4 mm $\varnothing$ wire, ii) a fluttering, but immobile moth due to a thicker 1.0 mm $\varnothing$ wire, iii) a non-fluttering but movable moth via a pulley system and iv) a non-fluttering and immobile moth. For more details, see Section 3.3.

To record the calls of the bats, we placed four custom-made ultrasonic microphones (Animal Physiology, University of Tübingen, flat frequency response within 6 dB from 20 kHz to 200 kHz) beneath each target and recorded all four channels simultaneously with a 600 kHz sampling rate using an analog-digital converter (Ni-USB 6356, Na-

tional Instruments). The converter was connected via USB to a laptop and signals were recorded with the software AVISOFT RECORDER (AVISOFT, version 4.2.27). Video footage was registered separately with an infrared camera system (Sanyo BW CCD camera VCB-3572 IRP, Munich, Germany; Computar lens M0518, Düsseldorf, Germany; Sony recorder DCR-PC8E, Berlin, Germany; IR-illumination with a custom-made IR-strobe). The videos were recorded on tape and were afterwards digitized for storage and analysis. We synchronized video and audio by clapping in front of the camera after each event. All equipment was powered using a 12 V, 80 Ah, 600 A car battery.

We registered how many approaches the bats did to each of the treatments. We differentiated between approaches that ended in a catch of the moth and approaches that did not. In the latter, the bats seemed to inspect the moth thoroughly (which usually involved hovering right in front of it) but did not attempt to catch it in the end.

The recording sessions took between 30 and 40 minutes per bat (including acclimatization). No more than 6 bats were recorded each night. After the recording sessions, we brought the bats to the entrance of the cave and released them there.

3.3.1 Data Analysis

We analyzed the audio recordings with a custom-made software written in Python (BAT-Spy, <https://github.com/jfsehuanes/BATSpy>). For details on how we extracted call times and other call parameters, see Section 2.3.

After extracting the call times of each sequence, we performed a manual split of each sequence into a **search phase**, an **inspection phase**, and a **terminal approach phase**. These were defined as follows: in the **search phase** the pulse intervals ranged between 20 and 35 ms; the **inspection phase** was characterized by a clear reduction of both pulse intervals and the standard deviation between PIs (range between 15 ms - 22 ms) and finally, the **terminal approach phase** was characterized by the continuous decline in pulse intervals until about 6 ms.

3.3.2 Permutation test

To test if the preference for fluttering targets significantly deviated from chance, we conducted a permutation test. In this test, we simulated the distribution of approaches to the four types of targets under the null hypothesis that bats randomly approach targets

without preference. Each simulation involved the following steps:

1. We initiated each permutation by drawing arbitrary numbers between 1 and 4, corresponding to the four target types in our experiment. These numbers represent the target chosen by a bat within a single approach.
2. We repeated this drawing process 45 times for each permutation, which corresponds to the total number of observed approaches in our actual experiment.
3. We counted the number of times each target type (1, 2, 3, or 4) was selected in the simulated 45 approaches.
4. This procedure was repeated one million times to create a robust distribution of outcomes under the assumption of random target selection.
5. From this distribution, we determined the 95th percentile for the number of approaches to fluttering targets (targets i and ii combined) and the 5th percentile for the number of approaches to non-fluttering targets (targets iii and iv combined).
6. We then compared these percentiles to the actual observed number of approaches to each type of target in our study.

3.3.3 Monte Carlo Simulations

To quantitatively assess how varying echolocation strategies influence the likelihood of detecting a moth's acoustic glint, we performed two Monte Carlo simulations.

In the first simulation, we compared the **glint hit rate** for the echolocation behavior of three different FM bats species (*N. tumidirostris*, *Eptesicus fuscus* and *Micronycteris microtis*) while in search phase, as well as *N. tumidirostris*' echolocation behavior during the inspection phase. On each run, the simulation generated bat "echolocation" calls, considering following call parameters: mean pulse duration (μ_{PD}), mean pulse interval (μ_{PI}) and finally the jitter between pulse intervals (σ_{PI}), defined as the standard deviation between pulse intervals. For *E. fuscus* and *Micronycteris microtis* we used the echolocation behavior parameters during search reported by [Surlykke and Moss \(2000\)](#) and [Geipel et al. \(2013\)](#), respectively (see parameters in Fig. 3.8). Next, we generated the time windows where the wings of the fluttering insect would be positioned in such

a favorable angle, that if hit by a bat's call, they would produce an acoustic glint (a potential glint window). These time windows were estimated by combining different glint durations from 0.1 to 10 ms and insect flutter frequencies from 0.5 to 250 Hz. We then counted the number of bat calls that overlapped with a potential glint window within a 1 second time period for each of the simulated bats. An example displaying the overlap of call times with potential glint times is shown in figure 3.2. This process was repeated 10 000 times for each insect glint duration and flutter frequency combination. Finally, we averaged the glint hit rates and displayed them in a contour plot (see Fig. 3.8).

In each subplot we also displayed the actual glint hit rate in orange for the parameters of a Noctuidae moth, with a flutter frequency of 25 Hz and a glint duration of 3.12 ms. We estimated the flutter frequency of the Noctuidae moths we captured in Colombia from slow-motion videos we took using a cellphone camera (OnePlus 6, Shenzhen OnePlus Science & Technology Co. Ltd., China). The **glint duration (GD)** on the other hand, was estimated according to

$$GD = \frac{G_{ar}}{2 M_{wsa}} \cdot \frac{1}{ff_m}, \quad (3.1)$$

where G_{ar} was the glint's angular range which we set to 18° according to Grossetête and Moss (1998); M_{wsa} was the maximum wing stroke amplitude which we set to 120° also based on estimations from the slow-motion videos of the fluttering Noctuidae moths from our experiments; and finally ff_m which was the flutter frequency of the moth.

Using very similar methods, we performed a second Monte Carlo simulation, this time analyzing the glint hit rate given multiple combinations of pulse intervals and pulse durations from simulated bats. However, on this simulation we also looked at the time until the first glint was hit, averaged over all 10,000 simulations. For each run we generated the times for the insects' glints (always with a different random start) with a fix glint duration of 3.12 ms and an insect flutter frequency of 25 Hz, thus mimicking the flutter characteristics of the Noctuid moths from our experiment. The call times of bats had a jittering corresponding to 20% of the PIs.

We also defined a theoretical perceived glint rate as follows:

$$p_{gr} = \frac{GD + PD}{GI/PI}, \quad (3.2)$$

where GD is the glint duration, PD the bat's **pulse duration**, GI the time interval between acoustic glints of the insect ($1 / ff_m$) and PI the **interval between bat calls**.

For the second simulation, we also displayed the glint rates that would correspond to echolocation parameters during search behavior that we found in the literature for following bat species:

- *Eptesicus fuscus*: $PI = 122 \text{ ms} \pm 19 \text{ ms}$, $PD = 11 \text{ ms}$, ([Surlykke and Moss \(2000\)](#));
- *Pipistrellus stenopterus*: $PI = 102 \text{ ms} \pm 4.0 \text{ ms}$, $PD = 9.7 \text{ ms}$, ([Kingston et al. \(2003\)](#));
- *Micronycteris microtis*: $PI = 20.1 \text{ ms} \pm 1.5 \text{ ms}$, $PD = 0.2 \text{ ms}$, ([Geipel et al. \(2013\)](#));
- *Myotis nattereri*: $PI = 46.4 \text{ ms} \pm 8.9 \text{ ms}$, $PD = 1.4 \text{ ms}$, ([Siemers and Schnitzler \(2004\)](#));
- *Phyllostomus discolor*: $PI = 30 \text{ ms} \pm 5 \text{ ms}$, $PD = 0.5 \text{ ms}$, ([Baier and Wiegbe \(2018\)](#));
- *Rhinolophus ferrumequinum*: $PI = 78.2 \text{ ms} \pm 9.9 \text{ ms}$, $PD = 53.5 \text{ ms}$, ([Jones and Rayner \(1989\)](#)).

Lastly, it is important to mention that for both simulations we assumed a conservative approach in which only one acoustic glint is present per wing flutter of a moth, although there are cases where 2 or more glints can occur per wing flutter, depending on the insect's specific wing movement pattern, as well as the ensonifying angle to the insect wings (for details, see [Kober \(1988\)](#), [Kober and Schnitzler \(1990\)](#)).

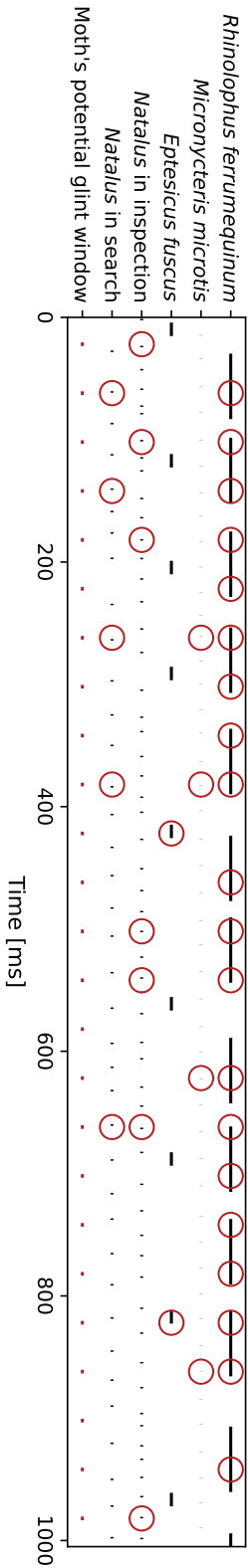


Figure 3.2: **Example simulation of how many potential glint windows overlap with calls from different bats using different echolocation strategies.** This figure shows different echolocation strategies of different bats during a 1 second length period. Each black line represents a bat's call and the line's length represents its duration. Bat calls that overlap with a Noctuidae moth's potential glint window (bottom row of red lines) are marked with red circles. The echolocation behavior of all bats corresponds to the search behavior, except for the "*Natalus in inspection*" row (for details on the echolocation parameters used, see Section 3.3.3).

3.4 Results

3.4.1 Preference for fluttering prey

We investigated the prey selection preference of *Natalus tumidirostris* bats in controlled semi-natural conditions in a flight-tent by presenting individuals with both fluttering and non-fluttering moths. From the 39 *Natalus tumidirostris* captured and released in the flight tent, only 19 bats performed at least one approach towards one of the target moths. All further results derive from the behavior of those 19 bats.

N. tumidirostris demonstrated a strong preference towards fluttering prey, with a total of 45 approaches recorded to fluttering targets of which 11 resulted in a successful catch (see Fig. 3.3). In contrast, only two bats performed an approach without a catch to one of our non-fluttering controls, which was an inert non-fluttering moth that we moved using a tethered pulley system (see Section 3.3). Furthermore, those two bats that approached the non-fluttering target iii (Bat 2 & 18), approached fluttering moths more often than non-fluttering moths (2 vs 1 each; see Fig. 3.3B). Approaches with catches were not recorded to any of the non-fluttering targets, thus highlighting the bats' strong selectivity on prey exhibiting fluttering motion.

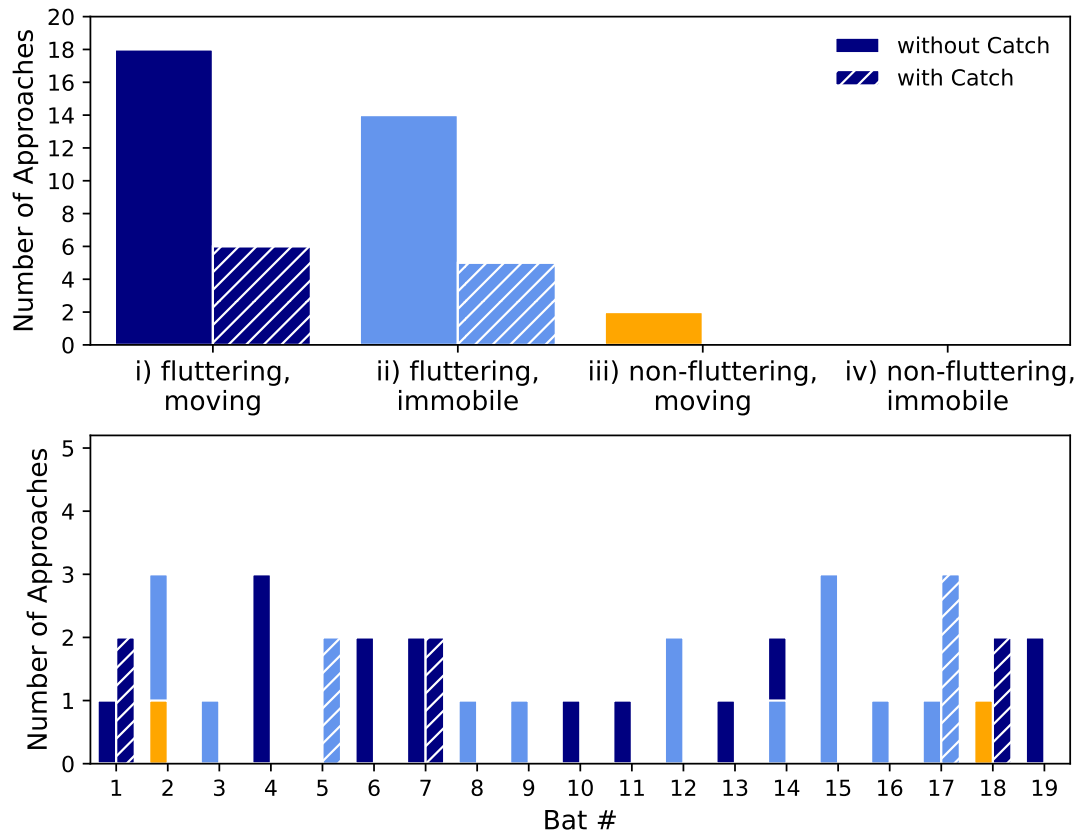


Figure 3.3: *N. tumidirostris* preference for fluttering prey. The upper barplot shows the cumulative number of approaches to each of the target moths; the lower plot shows the number of approaches of each individual bat as stacked columns (one for approaches with and another for approaches without catch). Bars with solid color represent the approaches without catch, while dashed bars represent those approaches where the moth was caught. Note that none of the bats caught any of the non-fluttering moths (no dashed yellow bars).

To test if the preference for fluttering targets was significantly different to what we would expect from chance, we performed a permutation test in which we randomly assigned approaches to each of the four possible targets (for details, see Section 3.3.2). When comparing the counted approaches from our observations with the corresponding counts from the permutation test, we observed a significant difference in all of the four targets ($p\text{-value} < 0.05$), i.e. our observations of approaches to fluttering prey were higher than expected by chance and the number of approaches to non-fluttering prey

were lower to what is expected from chance (see Fig. 3.4). This significant difference was valid both when looking at targets individually (Fig. 3.4 A) or when grouping the targets in fluttering vs non-fluttering targets (Fig. 3.4 B).

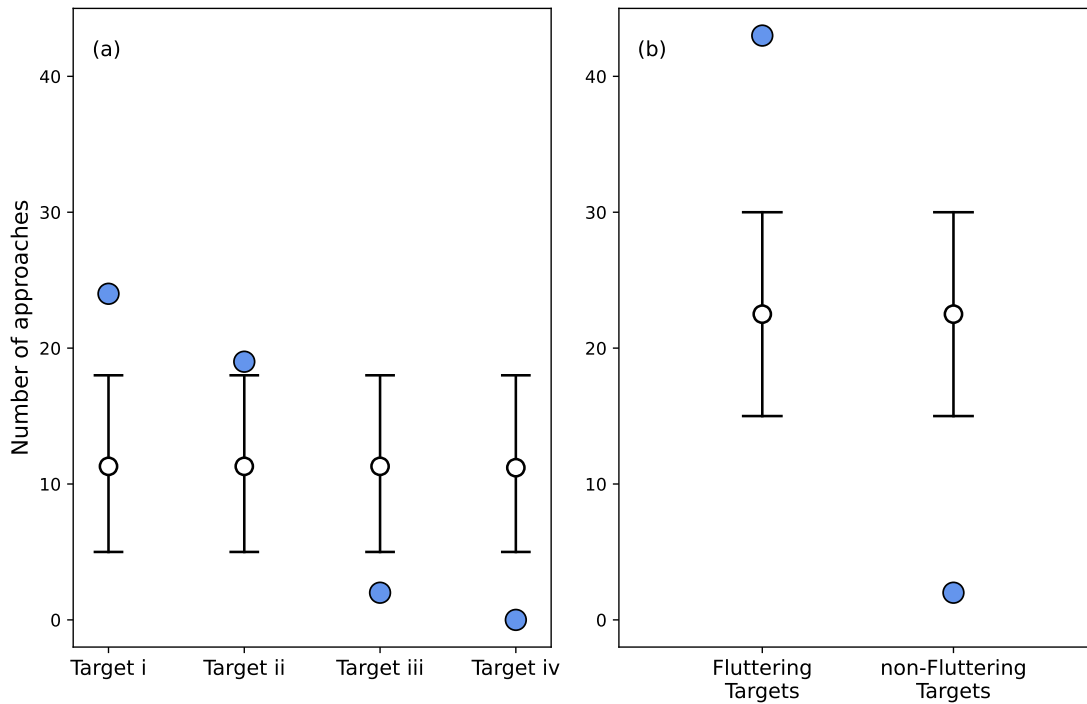


Figure 3.4: **Permutation test to verify preference for fluttering prey.** This figure shows the results of the permutation test done for each target individually (a) and after grouping the approaches into fluttering targets (i + ii) and non-fluttering targets (iii + iv) (b). The white circles show the median and the whiskers represent the 5th and 95th percentile of the permutation test, while the blue circles show the total number of approaches we observed from our bats to each of the targets. For more details, see Section 3.3.2.

3.4.2 Evidence for foraging in cluttered environments

On the night of the 13th of February 2020, we modified the flight cage environment by introducing clutter, such as leaves and branches in the walls and ceiling of one of the upper corners of the cage, to mimic a forested habitat. Also, instead of releasing a bat individually, we released three bats inside the flight cage at the same time. We then hung one fluttering moth nearly 10 cm equidistantly away from the left, right and upper sides

of the flight cage's cluttered corner. We observed that the bats were also successful in locating and capturing moths at this distance from clutter. We registered a total of 6 approaches (4 of them with catch) to fluttering moths. Note that no approaches were recorded to moths while they were not fluttering. These observations serve as evidence that *N. tumidirostris* can effectively hunt in densely cluttered environments.

3.4.3 Echolocation behavior analysis

Besides registering the number of approaches to the different targets, we were able to record echolocation calls on 40 of the 45 approach sequences. Each sequence was split into a **search phase** where pulse intervals (PIs) ranged between 20 ms and 35 ms with a mean of 27.5 ± 4.9 ms; an **inspection phase** defined as a clear reduction of the PIs with a mean of 17.8 ± 4.4 ms (range between 15 – 22 ms); and a **terminal approach phase** where the PIs descended constantly until about 6 ms. Four example approach sequences with their corresponding phases are shown in Fig. 3.5. The distributions of PIs from different phases were highly significant from one another (Mann-Whitney U test, p -value < 0.001 ; Fig. 3.6 (a)). The average duration of the inspection phase was 0.6 s (range between 0.2 s and 2.5 s), with shorter (though not significant) durations for approaches that preceded a catch (see Fig. 3.7). Not every approach contained all three phases, though we observed that 35 of 40 approaches contained an inspection phase. Also, we registered that 3 of the 29 recorded approaches without a catch contained a terminal approach, which is a behavior rather observed right before a catch. In fact, we observed a terminal approach phase in all recorded approaches that ended in a catch.

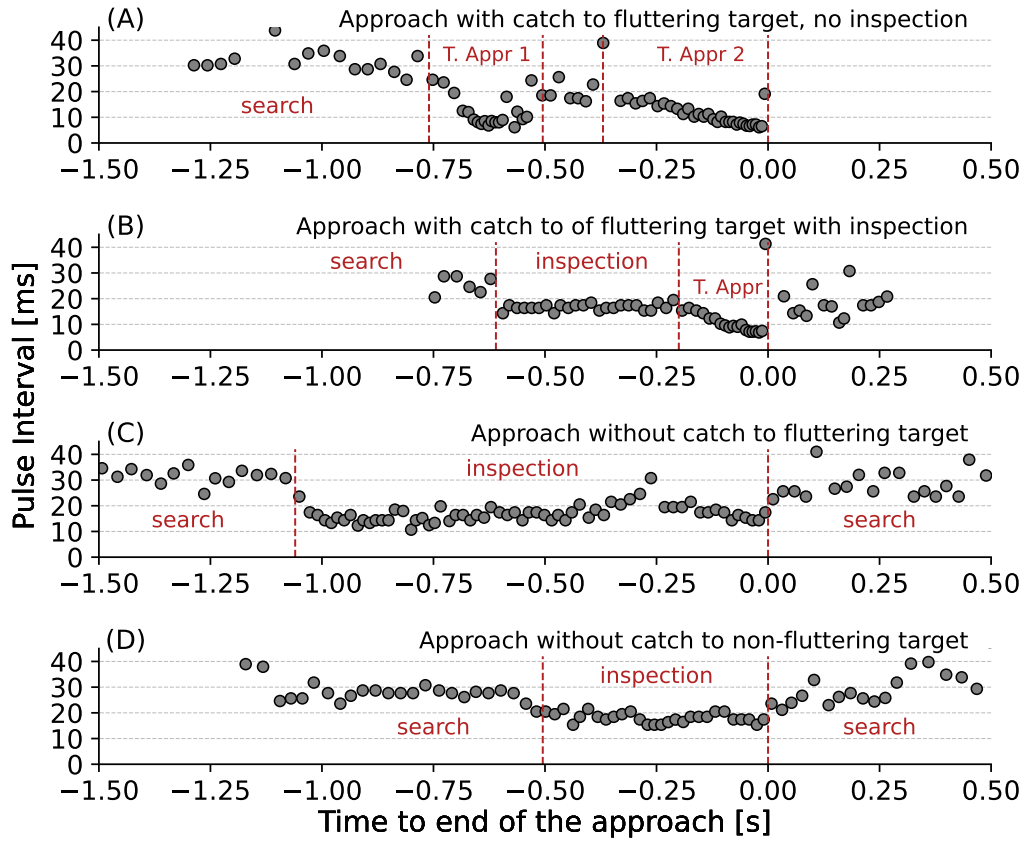


Figure 3.5: **Examples of the echolocation behavior while approaching to different targets.** Each subplot shows the time interval of the calls plotted over time. The time 0 in the X-Axis corresponds to the time of the shortest pulse interval in the sequence, which for (A) and (B) corresponds to the end of the terminal approach. The different echolocation phases are labeled in red. Note that in (B), (C) and (D), all inspection phases have a similar echolocation behavior in which the pulse intervals stabilize between 15 ms and 22 ms. (A) represents a sequence where the bat did not perform an inspection phase.

As we had already observed in previous recordings (see Section 2.4), the echolocation calls of these bats were extremely faint. This issue caused inaccuracies when extracting call parameters such as call durations and frequencies (see Section 2.3). Nonetheless, we compared call with relatively good signal-to-noise ratios, which corresponded to 296 search calls, 183 inspection calls from inspections where no moths were caught, 50 inspection calls from inspections that ended in a moth catch and 21 calls corresponding to the terminal approach right before a catch. The call duration was significantly shorter during inspections without catch than for inspections preceding a catch or than calls during search phase (Kruskal-Wallis & Dunn Test, $p\text{-value} < 0.001$; see Fig. 3.6 (b)). Also, the frequency bandwidth was higher for both inspection phases and the terminal approach phase, when compared to the search phase (Kruskal-Wallis & Dunn Test, $p\text{-value} < 0.001$; see Fig. 3.6 (c)). It is important to mention that this increase in frequency bandwidth may be explained by the proximity of the bats to the microphones during inspections and terminal approaches in comparison to calls recorded from the search phase.

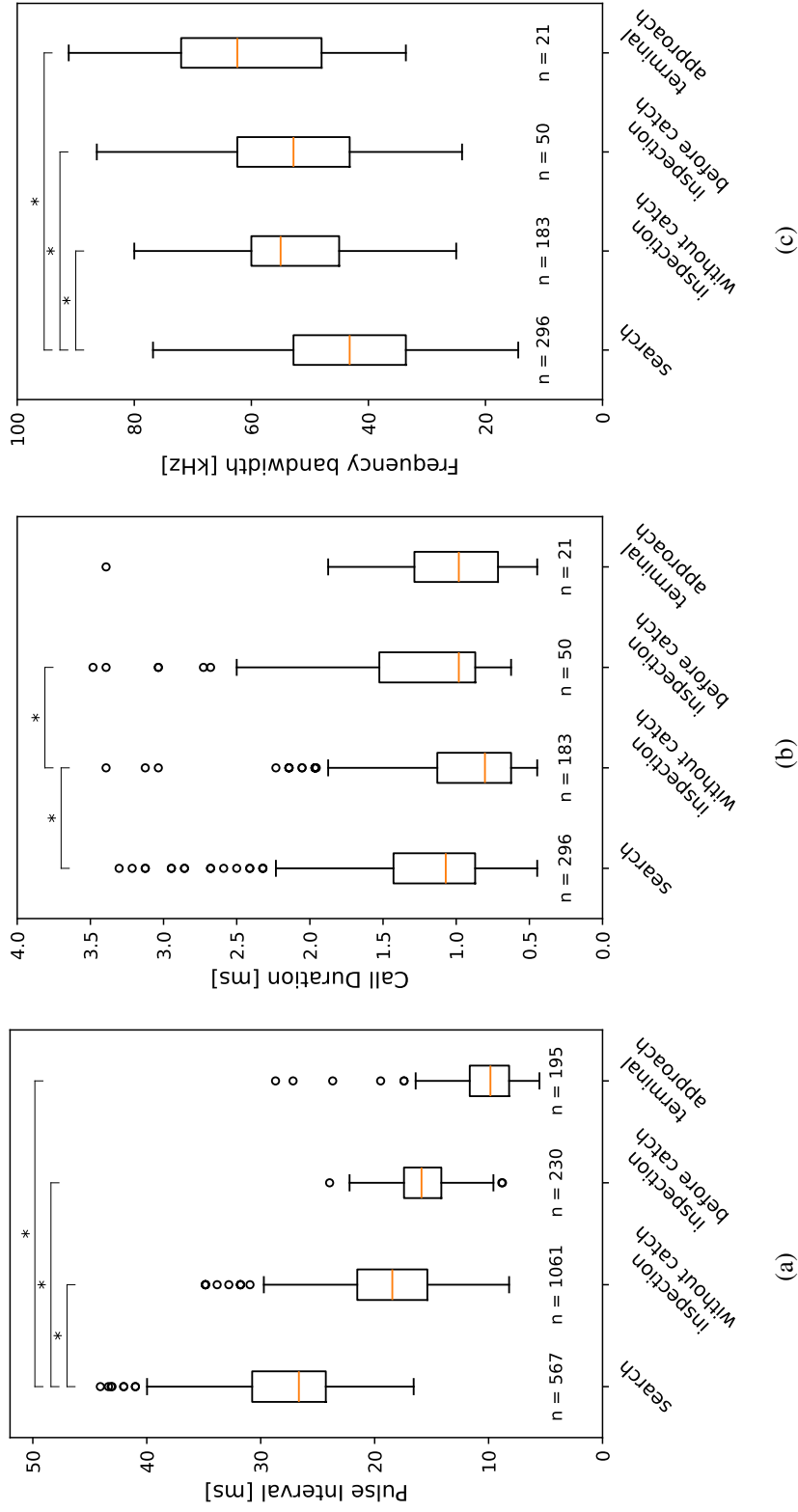


Figure 3.6: **Call parameters throughout different echolocation phases.** These plots show the parameters of calls with good signal to noise ratio during the 40 approach sequences we were able to record. (a) shows the pulse intervals, (b) shows the call duration and (c) shows the frequency bandwidth. Note that a single approach sequence does not necessarily contain all phases (for more details, see Sections 3.3.1 & 3.4.3). An * denotes a significant difference between categories.

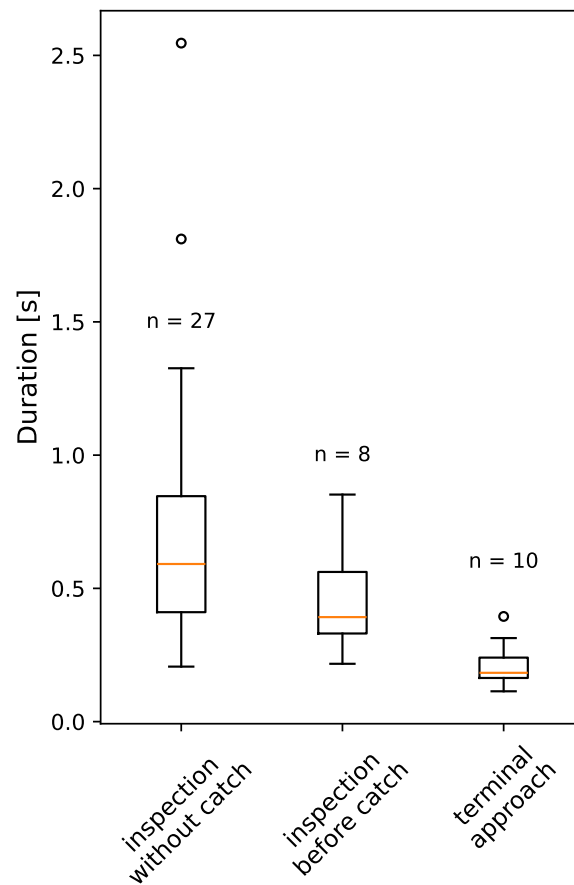


Figure 3.7: **Duration of inspection and terminal approach phases.** This figure displays the distribution of individual durations of the inspection phases and terminal approach. Note that most inspections are between 0.5 and 1. seconds long. Also, note that inspections after which a catch of a moth followed tend to be shorter.

3.4.4 Acoustic glint detection analysis

To better understand the effectiveness of *Natalus tumidirostris*'s echolocation strategy, specifically in terms of how many of its calls would overlap with a potential glint window, we conducted a Monte Carlo simulation. This simulation assessed the **glint hit rate**, considering a wide range of possible insect flutter frequencies and acoustic glint durations (for details, see Section 3.3.3). These simulations suggest that for the Noctuid moth species our bats were hunting during our experiments (flutter frequency = 25 Hz; glint duration = 3.12 ms), *N. tumidirostris* should be able to detect on average 4 glints per second using its continuous echolocation strategy during the search phase (see Fig. 3.8 A). This rate went up to 6 glints per second if *N. tumidirostris* switched to its echolocation behavior during the inspection phase thanks to shorter pulse intervals and a reduced standard deviation between pulse intervals (see Fig. 3.8 B). We also extended our simulation to examine the glint hit rates during the search phase for other FM bat species, estimating that both *Pipistrellus stenopterus* and *Micronycteris microtis* would hit 4 glints per second. *Phyllostomus discolor* and *Eptesicus fuscus* each registered 3 glints per second, while *Myotis nattereri* should hit an average of only 2 glints per second, as indicated in Fig. 3.9 A. Remarkably, the CF bat *Rhinolophus ferrumequinum* distinguished itself by registering an impressive 18 glints per second, thereby showcasing the highest potential glint hit rate among the species examined.

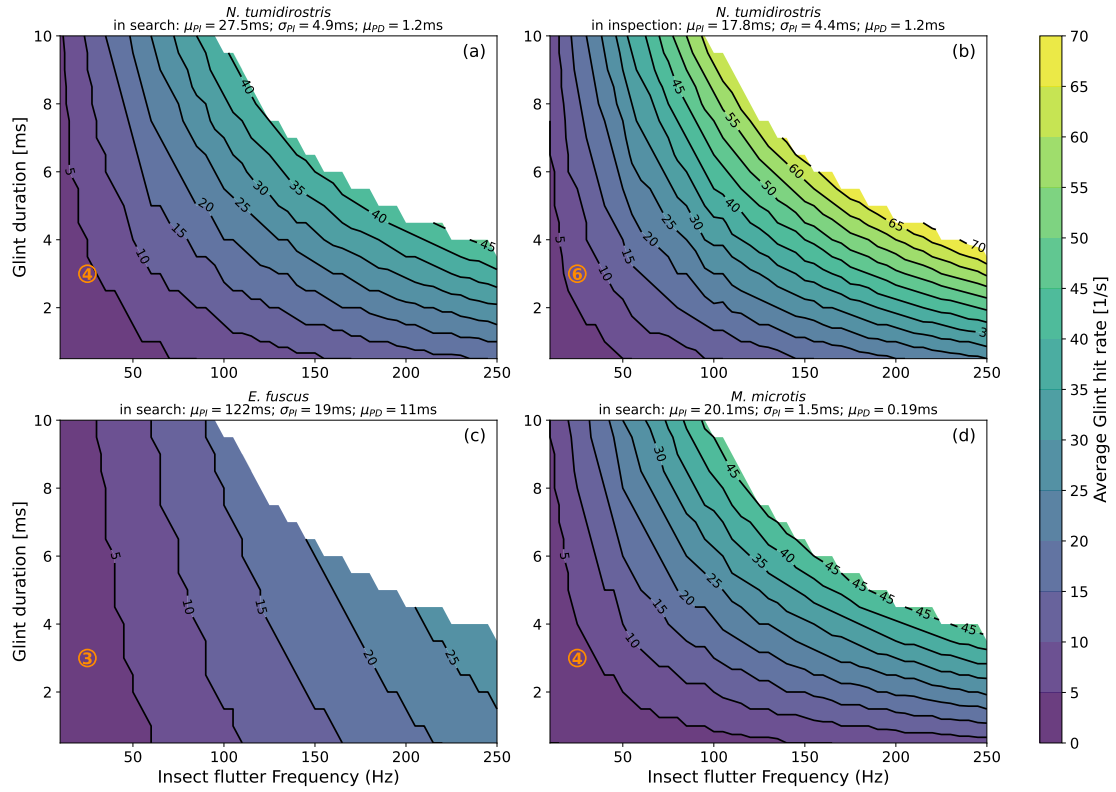
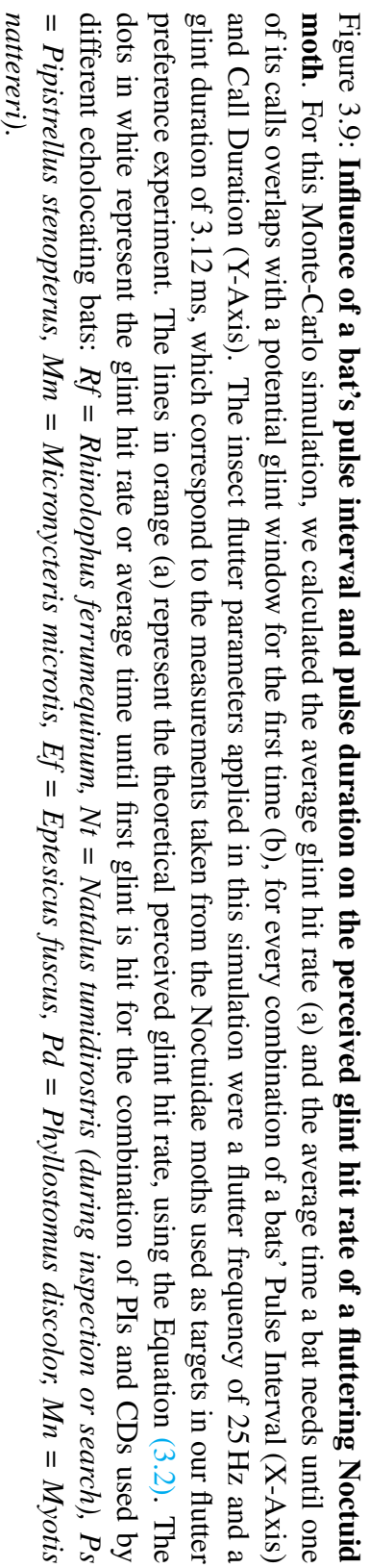


Figure 3.8: Influence of insect flutter frequency and glint duration on the perceived glint hit rate for different FM bats. This figure shows the result of 4 Monte-Carlo simulations (for details, see Section 3.3.3), each using a different set of bat echolocation parameters, which are shown in the upper part of each plot. Given a bat's echolocation parameters, we calculated the average glint hit rate, i.e. the amount of times a bat's call overlapped with an potential glint window of a fluttering insect, given a specific combination of flutter-frequency (X-Axis) and glint duration (Y-Axis). The numbers in orange shows the glint hit rate corresponding to a flutter frequency of 25 Hz and a glint duration of 3.12 ms, which were the parameters we determined for the Noctuidae moths we used as targets in the flutter preference experiment. Note that for *N. tumidirostris*, it should be to distinguish between two insects of different flutter frequencies when switching from search to the inspection behavior (compare (a) with (b)).

Additionally, we conducted a second Monte Carlo simulation, this time comparing a certain combination of possible pulse intervals and pulse durations produced by bats. We estimated both the glint hit rate and the average time until the first bat call would overlap with a glint, using the flutter parameters of a Noctuid moth (flutter frequency = 25 Hz; glint duration = 3.12 ms) throughout this simulation. First, we observed that our

simulation was in line with the theoretical equation we defined for the perceived glint rate (see (3.2); compare the values of the solid red lines with the simulations' contour colors of the average glint hit rate in Fig. 3.9 A). Additionally, we estimated that *N. tumidirostris* would need on average 206 ms to overlap an echolocation call with a glint from a Noctuidae moth during the search phase (see Fig. 3.9 B). This time went down to 148 ms for *N. tumidirostris* using its echolocation parameters during an inspection phase. In comparison, all other FM species examined required more time to hit a glint with their echolocation calls: *P. stenopterus* needed 219 ms, *M. microtis* needed 225 ms, *E. fuscus* needed 254 ms, *P. discolor* 260 ms, and *M. nattereri* needed 310 ms. Only the CF bat *R. ferrumequinum* demonstrated a substantially shorter average time of 39 ms to hit a glint.



3.5 Discussion

The aim of this study was to proof, whether the echolocation system of *Natalus tumidirostris* is adapted to detect and evaluate flutter information in the returning echoes of their very faint and steeply frequency modulated calls, which are emitted in a nearly continuous manner at low pulse intervals (see Section 3). In a behavioral study, we evaluated the preference of *N. tumidirostris* towards fluttering vs non-fluttering moths, while looking at the echolocation behavior during search, inspection, and final approach to their prey. We observed a strong preference of these bats towards fluttering prey items, that differed significantly from chance (Fig. 3.3 and Fig. 3.4). Out of the total 45 approaches we registered, 43 were directed towards fluttering moths, including all approaches that ended in a successful catch. No approaches were observed to moths while they were not fluttering aside from the 2 inspections towards the non-fluttering but moving prey (target iii in Fig. 3.1), yet without any attempt to catch the moth. These results evidence an indisputable preference towards fluttering prey and we conclude that *N. tumidirostris* must rely on the encoded flutter information in returning echoes from fluttering insects. This conclusion is also supported by previous observations in which these bats also performed approaches towards a stationary rotating blade and changed their echolocation behavior from search phase to inspection phase when doing so (see Section 2.4 and Fig. 2.4).

Whether bats that emit short frequency modulated calls with a low duty cycle, s.a. *N. tumidirostris*, can use flutter information when foraging in narrow spaces has been under debate. The duration of a single call is often too short to cover an insect's entire wing beat cycle (Grossetête and Moss (1998)) and the sampling rate during search behavior is usually not high enough to estimate an insect's wing beat frequency based on the Nyquist theorem (Nyquist (1928); Fontaine and Peremans (2011)). However, several studies have shown that flutter information is indeed available in FM echoes returning from fluttering insects. Ensonification of fluttering insects using short FM signals and a repetition rate of 200 Hz (Kober and Schnitzler (1990)) or 33 Hz (Moss and Zagaeski (1994)) revealed distinct changes over time in the amplitude and in the dominant frequency band of consecutive returning echoes depending on the relative timing of the position of the insect's wing and the timing of the impinging sound waves.

However, flutter information is not only encoded in succeeding echoes but also within a single glint echo. Behavioral experiments on *Pipistrellus stenopterus* (Sum and Menne (1988)) and *Eptesicus fuscus* (Roverud *et al.* (1991); Grossetête and Moss (1998)) show

that both FM bats were able to discriminate between two artificial blades rotating at different frequencies. A conclusion drawn from these results is that an echo returning from a fluttering target is composed of two elements: one consisting of a Doppler-shifted echo from the moving parts of the blade and the other consisting of a non-Doppler-shifted echo from the stationary parts of the rotating device. The interference pattern between these two waves creates a two-wavefront echo containing conspicuous frequency notches that could be easily discerned by the bat (Sum and Menne (1988); Mogdans and Schnitzler (1990); Schmidt (1988); Simmons *et al.* (1974); Simmons *et al.* (1990)). Such an interference pattern between stationary and moving parts is not exclusive of a rotating device in an experimental setup. Overlapping echoes from an insect's body (stationary part) and its fluttering wings (moving part) can create a similar two-wavefront interference pattern (see Moss and Zagaeski (1994); Grossetête and Moss (1998)). This naturally leads us to question how *N. tumidirostris*, with its unique echolocation behavior characterized by a continuous emission of calls with short pulse intervals of 27 ms, is able to detect and characterize prey items from those complex echoes. In particular it is important to discuss whether *N. tumidirostris* is decoding flutter information from a single glint echo, or a sequence of succeeding echoes, or both.

3.5.1 Decoding flutter information from glint rates

From our Monte Carlo simulations, we estimated that *N. tumidirostris*, while searching for prey, would hit on average 4 of the available 25 glints per second of a Noctuid moth (Mean PI: 27.5 ms, Mean PD: 1.2ms; see Fig. 3.9 A) and would need on average about 206 ms to hit the first glint. Given the very high frequency and very low source level of its calls, we expect the maximal prey detection distance of *N. tumidirostris* to be very short, and in consequence, there is most likely not enough time to evaluate the complete glint pattern of an insect in succeeding echoes at these rather low glint hit rates. Therefore, we conclude that during the search phase only very few or even a single echo containing a glint from a fluttering insect and thus the spectral information within a single echo may be sufficient for *N. tumidirostris* to create a hypothesis that there is potential prey nearby and then trigger a change in the echolocation behavior to confirm this.

We observed that before proceeding with the final approach towards a moth, *N. tumidirostris* transitions into an average 0.6 s long inspection phase (range: 0.2 s - 2.5 s) in which it reduces its pulse intervals to 17.8 ms on average (see Fig. 3.7 and Fig. 3.6a).

This corresponds to a nearly 56 Hz sampling rate, which, according to the Nyquist theorem, should be high enough to detect the 25 Hz flutter frequency of a Noctuid moth (Nyquist (1928), Shannon (1949)), but raises the question whether the inspection phase window of 0.6 s is long enough to do so. Another important aspect to consider is how *N. tumidirostris* could estimate higher flutter frequencies which are undersampled e.g. of insects such as Diptera, which can reach up to 100 Hz or more.

Contrary to the suggestion by Moss and Zagaeski (1994) that bats might synchronize their emission rate with the wingbeat rate of their prey to utilize a stroboscopic effect for determining the insect's flutter frequency, our observations indicate the absence of such behavior in *N. tumidirostris*. During the inspection phase, the bat's sampling rate of 56 Hz clearly exceeded the wingbeat rate of the prey, suggesting there must be an alternative mechanism at play for wing beat frequency estimation.

Our Monte Carlo simulations, however, propose a possible mechanism to roughly estimate the flutter frequency: a simple integrator that counts the number of detected glints per unit of time. Figure 3.8 shows that the average glint hit rate changes for different combinations of insect flutter frequencies and glint durations. In general, we can observe that the higher the flutter frequency of the insect, the higher the glint hit rate. Also, the increased sampling rate of the bat during the inspection phase enhances the resolution needed to discriminate between 2 different flutter frequencies (compare the much higher maximum glint hit rate of Fig. 3.8 B compared to A). However, it is important to criticize the fact that an average inspection length of 0.6 s may be a very limited time for such a mechanism to work. It is also important to highlight that the results of our simulations only display the average glint hit rate. The glint hit rate varies significantly depending on whether the emission rate is completely in phase with the wingbeat of the insect or not in phase at all, in which case the glint hit rate would be 0.

A much more interesting and plausible mechanism was proposed in a study published in 2011, where authors Fontaine & Peremans discussed the possibility of bats being able to determine an insect's flutter frequency with short FM signals using **compressive sensing (CS)** (see Fontaine and Peremans (2011), but also Candès (2006) & Donoho (2006)). CS deals with “the recovery or characterization of sparse signals from highly incomplete or highly sub-sampled data sets” (Fontaine and Peremans (2011)) and so offers a powerful framework for understanding how *N. tumidirostris* might efficiently process echo sequences to detect and characterize prey. CS operates under the assumption that the sig-

nal of interest is sparse, meaning that it has significant information concentrated in a few non-zero coefficients after being transformed to a more suitable basis. This assumption would enable bats using CS to represent an insect's flutter frequency accurately using far fewer samples than what the sampling theorem requires, effectively bypassing the need for high-frequency sampling.

This approach suggests that bats could employ a form of sophisticated biological signal processing, enabling them to identify and classify prey based on minimal, yet highly informative, acoustic signals. Insights from their experiments indicate that a bat employing an emission rate of 60 Hz—comparable to the 56 Hz observed in *N. tumidirostris* during its inspection phase—could reliably estimate a rotation frequency of 25 Hz within a mere 0.5 s sequence duration (refer to the plot in Fig. 8 of Fontaine and Peremans (2011) under the “Field sampling strategy”, which resembles *N. tumidirostris* the most). Furthermore, the data presented in that figure suggest the potential for *N. tumidirostris* to estimate flutter frequencies up to, or perhaps exceeding, its call emission rate of 56 Hz during the inspection phase. It is noteworthy, however, that the 2011 study of Fontaine & Peremans does not delve into the specifics of how CS might reconstruct flutter frequencies surpassing the emission rate, indicating the need for additional focused research in this area to fully grasp the scope of CS in FM bat echolocation. What the authors do highlight is that CS is a biological plausible system in bats: by drawing upon the findings of Condon *et al.* (1994), which illustrate the capability of neurons in the Inferior Colliculus to encode modulations similar to those from fluttering targets, Fontaine & Peremans effectively anchor the concept of CS within a biological framework, suggesting that such a sophisticated signal processing mechanism could indeed be naturally embodied in the auditory system of *N. tumidirostris*.

3.5.2 Comparison to other species

Interestingly, our Monte Carlo simulations show the glint hit rates during search for other FM bats were very similar to that of *N. tumidirostris*: *Pipistrellus stenopterus* and *Micronycteris microtis* also had 4 glints per second, while *Phyllostomus discolor* and *Eptesicus fuscus* had 3 glints per second. The other FM bat species we compared (*Myotis nattereri*) hit on average only 2 glints per second (see Fig. 3.9 A). These species also needed more time than *N. tumidirostris* until a glint was hit (see Fig. 3.9 B). We doubt however, that these species are able to evaluate flutter information to find insects.

Despite experimental evidence showing that *P. stenopterus* and *E. fuscus* can discriminate between targets of different rotating frequencies using physically rotating blades (Sum and Menne (1988), Roverud *et al.* (1991), Grossetête and Moss (1998)), it is crucial to consider that *P. stenopterus*, *E. fuscus*, and *M. nattereri* belong to the edge space foragers guild. Field recordings showed that *E. fuscus* utilizes pulse intervals of 130-150 ms during its search phase without engaging in an inspection phase before capturing prey (Surlykke and Moss (2000)). Additionally, Fontaine & Peremans' research on compressive sensing indicates that using search-phase echolocation parameters observed in the field, both *P. stenopterus* and *E. fuscus* would require over 2 seconds to accurately estimate an insect's flutter frequency—a period that significantly exceeds the brief duration of a complete approach sequence, which seldom surpasses one second (refer to Fig. 8 in Fontaine and Peremans (2011), under the "Field sampling strategy"). Both *E. fuscus* and *M. nattereri* are edge space foragers, primarily tasked with the detection and localization of prey through the evaluation of pulse-echo pairs, where prey echoes are distinct and not obscured by background noise (Denzinger *et al.* (2018)). While a glint echo could theoretically enhance insect detectability in edge or open spaces—being up to 20 dB louder than non-glinting insect echoes (Schnitzler and Denzinger (2011))—we argue that the search-phase echolocation behavior of these species is not optimally suited for reliably detecting and identifying fluttering insects.

Following with *Phyllostomus discolor*, it's pertinent to extend the skepticism towards the flutter detection capabilities of this species, considering its specific ecological niche and foraging strategies. Despite this species was able to discriminate modulated echoes from virtual targets (Baier and Wiegerebe (2018) & Baier *et al.* (2018)), *P. discolor* is an omnivorous bat and “most often a consumer of plant material with high levels of nectarivory” Kwiecinski (2006); (see also Dobat (1985)). This dietary preference suggests a limited necessity for a sophisticated echolocation system optimized for flutter detection in narrow spaces. Similarly, *Micronycteris microtis* specializes in hunting stationary prey like dragonflies and caterpillars on leaves (Kalka and Kalko (2006); Santana *et al.* (2011)). This bat species - which has been categorized to the active gleaners guild - ensonifies leaves in an oblique angle from above with ultra-short calls to discriminate leaves with prey from leaves without prey (Geipel *et al.* (2013); Geipel *et al.* (2019); Denzinger and Schnitzler (2013)). Both of these foraging methods, heavily reliant on detecting stationary or predictable targets rather than capturing fluttering insects, cast further doubt

on the necessity and development of advanced flutter detection mechanisms within these species.

In contrast, our simulations show that the well-studied flutter-detecting forager *Rhinolophus ferrumequinum* had a much higher average glint hit rate of 18 glints per second (see Fig. 3.9 A). Every single long constant frequency (CF) call of *R. ferrumequinum* hits at least one or sometimes even two glints (see Fig. 3.2). Furthermore, this CF bat needed on average 39 ms until one of its calls overlapped with a moth's glint (see Fig. 3.9 B). The use of flutter information for prey detection and classification has been shown for these narrow space flutter detecting foragers (for review see Schnitzler and Denzinger (2011)). The glint pattern contained in the echoes of these long signals encodes prey specific information on wing beat rate, wing size and aspect angle, which allows the discrimination of prey items (Von Der Emde and Menne (1989); Von Der Emde and Schnitzler (1990); Roverud *et al.* (1991); Koselj *et al.* (2011)). Flutter detecting foragers that operate with long CF calls have a highly specialized echolocation system to evaluate flutter information s.a. Doppler-Shift-Compensation and an auditory fovea (for review see Schnitzler and Denzinger (2011)).

3.5.3 Adaptive value of evaluating flutter information in confined spaces

We also demonstrated in this study that *N. tumidirostris* was able to find fluttering targets that were very close to clutter. These bats repeatedly detected and captured fluttering prey items as close as 10 cm from dense clutter. No approaches were observed to moths while they were not fluttering, thus suggesting that the bats must be relying on the information encoded in the returning echoes from fluttering insect wings to distinguish a prey from masking background echoes of densely cluttered spaces. In this experiment, the bats displayed a high maneuverability, showing no difficulties to navigate in the confined space of the corner of the flight cage, which on the night of the 13th of December 2020 included clutter. This reinforces our assumption that densely cluttered spaces are the foraging habitat of this species, similar to what has been observed for other Natalids (Miller (1904), Jennings *et al.* (2004), Sanchez *et al.* (2017), Torres-Flores and López-Wilchis (2018) and López-Wilchis *et al.* (2020)). All these results give together a very strong indication that *N. tumidirostris* can detect and possibly also evaluate flutter motion

and use this information to detect prey items in cluttered environments.

Bats foraging in narrow spaces face significant challenges as prey echoes may be masked by background clutter. Furthermore, they encounter echoes from numerous nearby objects, compelling them to engage in constant pattern recognition to determine whether the acoustic scene contains prey echoes or not ([Denzinger *et al.* \(2018\)](#)). In such environments, echoes from background objects can suddenly amplify when aligned perpendicularly to the bat's sonar beam, and multi-wavefront echoes can also arise from multiple overlapping background reflectors. A key distinguishing feature of a fluttering insect's glint echo is that it covers only a part of the echo and shifts across different frequencies in a successive train of echoes ([Kober and Schnitzler \(1990\)](#); [Moss and Zagaeski \(1994\)](#)). We suspect this quality can greatly distinguish an insect echo from other loud background echoes. Additionally, the spectral fine structure within a glint, arising from reflections off the insect's moving wings and body, may provide specific cues indicative of a fluttering target, especially during the inspection phase, where the glint hit rate is higher. *Natalus tumidirostris* exhibits remarkable adaptations for foraging within dense vegetation, characterized by its unique continuous echolocation behavior and additional morphological and acoustic modifications. These adaptations include the emission of extremely faint, high-frequency calls that effectively minimize clutter interference and wings designed for exceptional maneuverability, with a very low aspect ratio and low wing loading (Table [2.1](#)). Together, these traits equip *N. tumidirostris*—and likely other Natalids—with specialized echolocation capabilities for effectively detecting and capturing fluttering insects in highly cluttered environments.

Chapter 4

Diet analysis using metabarcoding for a bat colony of *Natalus tumidirostris*

** this chapter was carried out in close collaboration with the Zentrum für Molekularbiologie der Pflanzen (ZMBP) in Tübingen and **Manuel Fonseca**

4.1 Abstract

This study presents the first comprehensive diet analysis of the Trinidadian funnel-eared bat, *Natalus tumidirostris*, employing metabarcoding techniques to sequence mitochondrial DNA (**mtDNA**) from bat scat samples. The primary objectives were to ascertain the bat's feeding strategy, identify the arthropod community in its diet, and explore the link between its echolocation behavior and prey capture. Scat samples were collected from the Macaregua cave in Santander, Colombia and subjected to DNA extraction, PCR amplification, and high-throughput sequencing.

Our analysis identified 2703 Amplicon Sequence Variants (**ASVs**) from the scat samples, with Diptera and Lepidoptera being the most abundant orders. Surprisingly, the arthropod family with the highest abundance of ASVs was Tachinidae, a family of flies that parasitizes larvae of other insects. We discuss why the presence of Tachinidae flies is caused by secondary predation, leading to the conclusion that *N. tumidirostris* is a dedicated specialist of Lepidoptera, consuming both fluttering adult moths, as well as caterpillars. We considered the rest of arthropod families as fall-back-food items.

Additionally, this study sheds light on the bat's hunting strategies. Previously, this species was described to hunt in cluttered spaces using the flutter-detection strategy by

means of continuously sampling the environment with short and broadband frequency modulated calls (see Chapters 2 & 3). However, the evidence of non-fluttering insects like spiders and caterpillars in the diet suggest that *N. tumidirostris* must also use the ‘active gleaning’ strategy as an additional hunting strategy for foraging prey in cluttered spaces.

The findings reported in this study contribute as the first approach to understand the dietary habits and ecological role of *N. tumidirostris*, suggesting a specialization towards Lepidoptera of both moths and caterpillars.

4.2 Introduction

Prey and predator interactions shape the community ecology of many species. These interactions help to understand the strategies by which predators can capture their food items, especially in species that have specialized senses and cryptic behaviours such as some bat species (Wray *et al.* (2021)). Characterizing predator-prey interactions is far more challenging for generalist predators because they are able to shift their foraging strategies depending on factors such as prey availability, nutritional demands, energy requirements or preferred food items (Baudrot *et al.* (2016)). But obtaining detailed dietary information of generalist predators can be challenging, limiting our understanding of the role certain species play in their natural ecosystems.

This lack of dietary information is particularly prominent in bats, despite extensive research on their foraging behaviour and roles in food webs (Wray *et al.* (2021), Zeale *et al.* (2011)). We still lack detailed diet descriptions for many bat species. Mainly since observing the prey items these animals actually eat can be very demanding, if not impossible. Some studies have focused on inspecting visually stomach content or fecal material of bats as an approach to assess the dietary composition (Whitaker *et al.* (1996), Willig *et al.* (1993)). Nonetheless, these methods can be invasive and lack taxonomic resolution, leading to misleading conclusions about bats feeding ecology. This is not the exemption for the Trinidadian funnel-eared bat (*Natalus tumidirostris*) for which information about its biology is almost non-existent (Tejedor (2011)).

N. tumidirostris is a widespread species in the north of South America that inhabits different ecosystems such as dry cactus scrubs, wet forests and deciduous forests (Eisenberg (1989), for details see Section 2.2). Sister species from the Natalidae family

have been reported to feed on arthropods, as some stomach content analysis have shown (Silva Taboada (1979), Santos-Moreno and Soriano-Cruz (2019)). Also, a more detailed fecal analysis for *Natalus mexicanus* revealed a large amount of Araneae in the diet, besides Coleoptera, Diptera and Lepidoptera (Torres-Flores and López-Wilchis (2018)).

Recent studies show that *N. tumidirostris* possess a unique echolocation behaviour. It produces ultra-short, frequency modulated (**FM**) calls in an almost continuous fashion while searching for prey, which allows it to detect and hunt fluttering insects in densely cluttered spaces, such as the forest canopy (see Chapters 2 & 3). However, no observational studies have evaluated whether the diet of *N. tumidirostris* mainly consists of fluttering insects as one would expect, given its particular echolocation behavior or whether it also hunts for stationary non fluttering prey such as spiders as described for *N. mexicanus* (Torres-Flores and López-Wilchis (2018)). Techniques such as metabarcoding, which involves the use of genetic barcodes, polymerase chain reaction (**PCR**) and high throughput sequencing (**HTS**), can help to address the prey-predator dynamics of a species within ecosystems (Creer *et al.* (2016)). With this non-invasive technique we can identify multiple taxa in environmental samples (e.g. water, soil, scats) by amplifying short conserved gene sequences, running a parallel sequencing of PCR amplicons (Valentini *et al.* (2009)). Metabarcoding has been crucial in uncovering feeding ecology, food-webs, host-microbiota bonds and trophic interactions of different taxa of organisms such as bats, primates, crustaceans and bears (Siegenthaler *et al.* (2019), Zeale *et al.* (2011)).

In this study, we aimed to broadly characterize for the first time the diet of the bat species *Natalus tumidirostris* using the non-invasive technique metabarcoding. For this purpose, we focused on sequencing mtDNA remaining in the bats' scats that we used as barcodes to identify the prey this bat species eats. Specifically, we aimed to: (i) evaluate whether this bat species has a generalist or a specialist feeding strategy, (ii) assess the arthropod community in the scats of wild Trinidadian funnel-eared bats and (iii) understand the association between the echolocation behavior of *N. tumidirostris* and the prey they are able to detect and capture.

4.3 Materials and Methods

4.3.1 Collection of Samples

Scat samples were collected at the Macaregua cave, located in Santander, Colombia (N 06°39'36.2", W 73°06'32.3"), being perhaps the cave with the highest bat richness in Colombia. It inhabits 8 species that use the space permanently or sporadically, and populations range from 7.000 to 10.000 individuals (Pérez-Torres *et al.* (2015)). Scat samples were collected in 10 different nights during the months of October and November of 2018. We obtained the scats by setting a plastic net of 1 m² right below the *N. tumidirostris* roost and thereby avoiding any contact with the ground. The roost of this species is very conservative, and bats of this species always returned to this very same spot of the cave for roosting. Thanks to this we can be sure that our samples belong to *N. tumidirostris*. We collected fresh samples every night after the bats left the roost to avoid unnecessary disturbances. Neither sex, age or individual identification were accounted for when collecting the bat scats. Samples were transferred to 2 ml Eppendorfs with 90% ethanol in a 1:1 volume ratio proportion. We collected a total of 100 Eppendorfs with scat samples (10 per day). Afterwards, the samples were lyophilized and stored at -20°C at Universidad de Los Andes, Colombia before they were sent to Eberhard Karls Universität Tübingen in Germany.

4.3.2 DNA Extraction

DNA was extracted from each sample (N=100) using the Fast DNA Stool Mini kit following the manufacturer's protocol (QIAGEN). DNA yield was quantified using @Nano-Drop ND-1000, samples below 10 ng/μL were discarded and a new extraction of those scat samples was made. Samples were pooled at equimolar concentration into effective sampling days (N = 10); Pooling has been reported to reduce the molecular sequence richness of the samples, obscuring less frequent and rare prey DNA (Mata *et al.* (2019)). For this reason, we included two replicates of each pooled sample during the PCR procedures in order to detect less common prey sequences (Siegenthaler *et al.* (2019)).

4.3.3 PCR and Bioinformatics

Following DNA extraction, we amplified two complementary regions of the COI mitochondrial gene using two universal degenerated primers for arthropods ZBJ (Zeale *et al.* (2011)) (ArtF1c: 5'-AGATATTGGAACWTTATATTTTATTTTGG-3', ArtR2c: 5'-WACTAATCAATTWCCAAATCCTCC-3'); ANML (F: 5'-GGTCAAACAATCATAAAGATATTGG-3', R: 5'-GGWACTAATCAATTCCAAATCC-3') (Jusino *et al.* (2019)). For each pool we made two PCR replicates in order to increase the probability of detection of rare sequences within the samples. We prepared a PCR master mix for each primer of 10 μ L that included: 10 μ L of Platinum II Taq Hot-Start DNA Polymerase (Thermo Fisher ScientificTM), 0.8 μ L of 10 nM F primer, 0.8 μ L of 10 nM R primer, 7.4 μ L of Nuclease Free Water (Invitrogen) and 1 μ L of DNA. The ZBJ primer was amplified using the following Access Array cycling: 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 1 min at 45°C, 30 s at 72°C, followed by a final extension step of 72°C for 5 min. The ANML primer was amplified using the following Access Array cycling: 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 1 min at 46°C, 30 s at 72°C followed by a final extension step of 72°C for 5 min. PCR products were visualized on 2% agarose gels and were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions.

The library was built and sequenced at the Illumina MiSeq Nano v2 (2x150) platform at Zentrum für Quantitative Biologie (QBiC) in Tübingen, Germany. We included negative and positive controls among the sequenced samples. Sequences were demultiplexed, primers and adapters were removed from the sequences with the cutadapt tool. Sequence reads were processed using the DADA2 v1.18.0 package (Callahan *et al.* (2016)). Samples were joined by paired-ends (PE), dereplicated, and amplicon sequence variants (ASVs) were inferred by pseudo-pooling. Chimeric sequences were identified and removed. Afterwards, ASVs were assigned taxonomy using the built-in curated COI database with the software *AMPTk v 1.4.0* tool. We removed ASVs that matched with both negative and positive controls (a product of tag jumpings during the sequencing), as well as sequences below a 90% of match identity.

4.4 Results

We identified a total of 2703 Amplicon Sequence Variants (ASVs) in the scats of the *N. tumidirostris* colony of the Macaregua cave in Santander, Colombia. From the ZBJ primer, we obtained 1465 ASVs with an average length of 150 base pairs and 1228 ASVs with an average length of 180 bp from the ANML primer. After grouping the ASVs of both primers and assigning taxonomy up until species order, we observed two predominant orders in the diet of *N. tumidirostris*: Diptera with a relative abundance of 46.6% and Lepidoptera with 31.4% (see Table 4.1). Statistical analysis indicated that besides Diptera and Lepidoptera, also Hemiptera had significantly more ASVs than the rest of arthropod orders, with a relative abundance of 5.1% (ANML-primer: One-way-ANOVA $F=97.14$, $df=7$, $p\text{-value} < 0.001$; ZBJ-primer: One-way-ANOVA $F=205.2$, $df=7$, $p\text{-value} < 0.001$; see Fig. 4.1). Though not significantly more abundant than other orders, it is important to mention the presence of spiders with a relative abundance of 4.6%. Also, we observed that spiders were present throughout all sampling days (see Fig. 4.2)

Table 4.1: **Relative abundance of Amplicon Sequence Variants counts corresponding to the different arthropod orders present in the scats of *N. tumidirostris*.** This table shows the overall ASV counts of all 10 sampling days.

Class	Order	ASV Counts	Relative abundance [%]
Insecta	Diptera	1226	46.6
Insecta	Lepidoptera	826	31.4
Insecta	Hemiptera	135	5.1
Arachnida	Araneae	121	4.6
Insecta	Coleoptera	83	3.2
Insecta	Hymenoptera	67	2.5
Insecta	Blattodea	53	2.0
Insecta	Orthoptera	26	1.0
Insecta	Neuroptera	26	1.0
Insecta	Odonata	14	0.5
Others		54	2.1
Total		2631	100

When taking a finer taxonomic look at family level, we were surprised to find that 29.1% of the sequences corresponded to flies belonging to the Tachinidae family (see

Table 4.2), which is a group of parasitic flies. In contrast, the second most abundant Diptera family was Muscidae with only 2% relative abundance. The four main families of Lepidoptera we found were: Geometridae (7.6%), Erebidae (5.4%), Noctuidae (4.2%) and Tortricidae (2.3%). The most abundant Hemiptera family present in the diet was Cicadidae (3.7%).

We considered further taxonomic analysis beyond the family level useless because many sequences were matched with arthropod genera that we know are not distributed in the neotropics.

Table 4.2: Relative abundance of Amplicon Sequence Variants counts corresponding to the different arthropod families present in the scats of *N. tumidirostris*. This table shows the overall ASV counts of all 10 sampling days. Note that the lower total counts of ASVs compared to Orders in Table 4.1, is due to the impossibility to assign some sequences to any family present in the databases.

Class	Order	Family	ASV Counts	Relative abundance [%]
Insecta	Diptera	Tachinidae	542	29.1
Insecta	Lepidoptera	Geometridae	142	7.6
Insecta	Lepidoptera	Erebidae	101	5.4
Insecta	Lepidoptera	Noctuidae	79	4.2
Insecta	Hemiptera	Cicadidae	68	3.7
Insecta	Lepidoptera	Tortricidae	43	2.3
Insecta	Diptera	Muscidae	38	2.0
Arachnida	Araneae	Theridiidae	37	2.0
Insecta	Diptera	Syrphidae	34	1.8
Insecta	Lepidoptera	Nymphalidae	34	1.8
Insecta	Lepidoptera	Crambidae	32	1.7
Insecta	Lepidoptera	Gelechiidae	27	1.5
Insecta	Lepidoptera	Pyalidae	27	1.5
Insecta	Diptera	Sarcophagidae	26	1.4
Insecta	Lepidoptera	Hesperiidae	25	1.3
Insecta	Diptera	Culicidae	21	1.1
Insecta	Hymenoptera	Braconidae	18	1.0
Insecta	Diptera	Limoniidae	17	0.9
Insecta	Diptera	Drosophilidae	16	0.9
Insecta	Blattodea	Ectobiidae	15	0.8
Others			519	27.9
Total			1861	100

4.5 Discussion

In this study, we investigated the diet of the bat species *Natalus tumidirostris* using metabarcoding and high-throughput-sequencing (HTS) of scats from a colony roosting inside the cave Macaregua in Santander, Colombia. We found that the arthropod sequences identified in the scats were predominantly from Diptera and Lepidoptera, but also Hemiptera, Araneae and other insect orders were present to a lesser extent (see Table 4.1 and Fig. 4.1). Though metabarcoding and HTS are considered as semi-quantitative methods (Porter and Hajibabaei (2018), Deagle *et al.* (2019)), this diet analysis can give us a broad first picture of the arthropod community that *N. tumidirostris* is consuming.

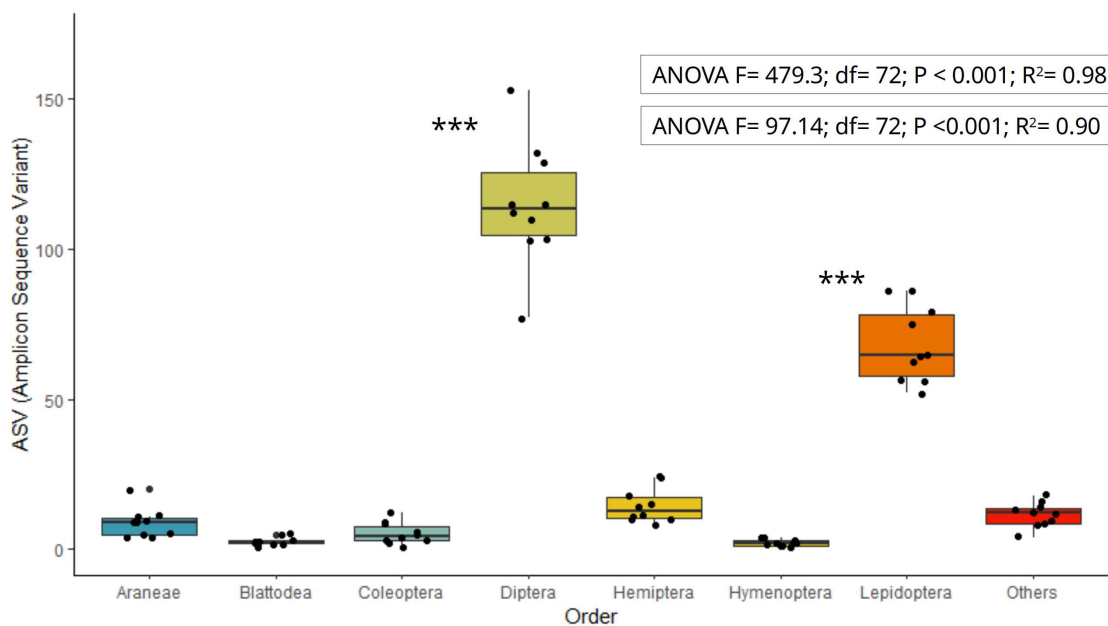


Figure 4.1: **Main arthropod orders in the diet of *N. tumidirostris*.** Each boxplot shows the distribution for the different arthropod orders present throughout the 10 sampling days. The dots represent the count of ASVs for each sampling day. Note that both Diptera and Lepidoptera have significantly more ASVs than the other arthropod orders. For details, see Section 4.4

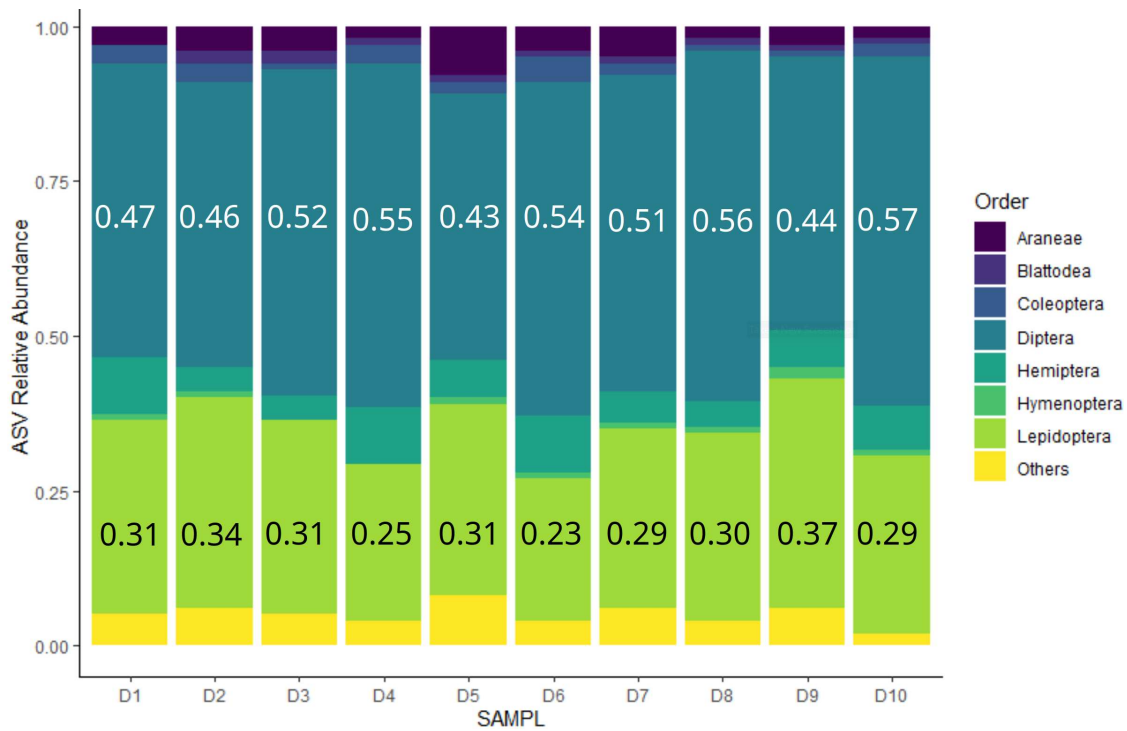


Figure 4.2: **Relative abundance of the arthropod diet of a colony of *N. tumidirostris* throughout 10 consecutive sampling days.** The main arthropod orders present in the diet are displayed. Note that spiders (non-fluttering prey) are present in all sampling days.

The most interesting result from this analysis is the predominance of sequences belonging to the Tachinidae family. Tachinids are diurnally active flies that parasitize other arthropods during the day and remain inactive during crepuscular or night hours (Stireman *et al.* (2006)). We thus argue that the large number of Tachinid sequences found in our samples are most probably caused by secondary predation; meaning that the colony of *N. tumidirostris* must be eating insects infected with tachinids. Tachinids are well-known parasites of Lepidoptera larvae as well as Hemipterans (Stireman *et al.* (2006), Tschorsnig (2017)), which are the other main taxonomic orders found in our samples. Furthermore, a single caterpillar may be parasitized by several tachinid species (Oberhauser *et al.* (2017), Stireman *et al.* (2006)), thus explaining why the number of Amplicon-Sequence-Variants (ASVs) belonging to the Tachinidae family is so large. The imagos of Tachinidae are diurnal (Pers. communication with Dr. James O' Hara). Though with our analysis methods we can't rule out that the bats could be directly pre-dating Tachinid flies resting on foliage, we argue this is very unlikely given that Tachinid

flies are rather small (range between 2 mm and 20 mm, [Stireman et al. \(2006\)](#)) and in contrast, larvae of Lepidoptera are a much more nutritious and suitable meal to fuel the high energetical demands of a bat's powered flight ([Thomas and Suthers \(1972\)](#), [Winter and Von Helversen \(1998\)](#)). A follow-up study that also includes traditional diet analysis methods, such as stomach content analysis or traditional feces analysis, should reveal or discard the direct predation of adult Tachinids by *N. tumidirostris*.

If we consider Tachinids (and in consequence nearly all the abundance of Diptera in the diet) are product of secondary predation, then the main arthropod order present in the scats of *N. tumidirostris* would be Lepidoptera with a relative abundance of more than 50%. Such high selectivity towards a single taxonomic group gives a strong suggestion for *N. tumidirostris* to be a dedicated specialist of Lepidoptera. A fact that supports this claim is that 9 of the most abundant 15 arthropod families found in the scats corresponded to the Lepidoptera order (see Table 4.2). Furthermore, traditional stomach content analysis for other Natalids also show a high abundance of Lepidoptera compared to other arthropod orders ([Silva Taboada \(1979\)](#), [Santos-Moreno and Soriano-Cruz \(2019\)](#)). Our results suggest that the rest of arthropod orders could be considered as fall-back-food items of *N. tumidirostris* diet; meaning that in cases where Lepidoptera abundance is low, the bats shift their diet to less preferred food items, s.a. Hemiptera or Araneae.

Since the methods used in this study only grant us a limited taxonomic resolution of analysis, future diet analysis should also include a local sampling of insects to create the libraries, particularly in the neotropics. This would allow us to make a direct comparison between the arthropod community present in the hunting habitat of *N. tumidirostris* with what they actually eat, similar to what Fonseca and collaborators did for their arthropod diet analysis on Woolly monkeys ([Fonseca et al. \(2022\)](#)). Ideally, one should carry out such a study over the course of an entire year to observe seasonal arthropod abundance patterns and how the bats react to them. In addition, it would be also interesting to evaluate the diet composition for different groups of the colony. For example, assess if females change their diet during pregnancy and lactation periods, or if juveniles have different feeding preferences than adults.

In previous studies, we demonstrated how *N. tumidirostris* can perceive insect flutter motion and use it to detect and hunt prey amidst cluttered environments (see Chapters 2 & 3). They emit broadband high frequency calls with a duration of about 2 ms in a nearly continuous fashion which short pulse intervals, adapted for glint detection with

FM signals at low duty cycle. The probability to perceive a glint depends besides other factors on the glint duration produced by the insect wing, as well as the insect wing beat. Due to their comparably low wing beat rate the glint duration in lepidoptera is rather long (see Chapter 3). This may explain the high selectivity for lepidoptera in the diet of *N. tumidirostris*. Additionally, the high abundance of tachinid sequences, which we assume are a product of secondary predation, indicates that caterpillars are also taken in a considerable amount. The presence of non-fluttering caterpillars and spiders in the diet of this species was rather unexpected. The only explanation for this is that, besides the flutter-detecting strategy, *N. tumidirostris* also relies on the ‘active gleaning’ strategy to find prey in cluttered environments. Active gleaners find their prey by thoroughly scanning single plants in search for arthropods resting on leaves (Geipel *et al.* (2013), Geipel *et al.* (2019), Denzinger and Schnitzler (2013)). They approach each leaf with an optimal oblique angle, thereby using the leaf as a “specular reflector” and so determine if there is prey resting on it or not (Geipel *et al.* (2013), Geipel *et al.* (2019), Denzinger and Schnitzler (2013)). *Micronycteris microtis*, the so far only known active gleaner, has been described to “mainly prey on large insects, including beetles (Coleoptera), katydids (Orthoptera), caterpillars and moths (Lepidoptera), cicadas (Cicadina)” (Geipel *et al.* (2013)). Since *N. tumidirostris* can also hover in confined spaces (see Chapters 2 & 3), we consider it very likely that this species could also hover in front of leaves in search for prey, just like *M. microtis*. Furthermore, the echolocation calls of *N. tumidirostris* have similar properties to those of *M. microtis*: they are both short (Nt: 2ms vs. Mm: 0.2 ms), high frequency (Nt freq. range: 152.6 – 105.1 kHz vs. Mm freq. range: 143.3 – 68.6 kHz) and have a large bandwidth (Nt: 48.5 kHz vs. Mm: 83.4 kHz). The active gleaning strategy may also explain the presence of predominantly diurnally active arthropods such as Nymphalidae (imagos or larvae) in the prey spectrum of *N. tumidirostris*.

So far, the only study on the echolocation behavior of *N. tumidirostris* when searching and catching prey has been carried out while offering them fluttering moths (see Chapters 2 & 3). How these bats forage for stationary, non-fluttering prey items is completely unknown. Caterpillars might be caught when sitting on leaves but also when hanging on silk threads between twigs. Also, spiders might be caught sitting in their webs or hiding under leaves. Since it is still not yet understood how bats are able to detect spiders on spider webs, a future study ensonifying spider webs with and without a spider using echolocation sequences of *N. tumidirostris* may shed more light about the information

contained in these kind of echoes. Further studies on the foraging and echolocation behavior are necessary to fully understand the sensory ecology of *N. tumidirostris*.

Chapter 5

Conclusions

This dissertation provides novel insights into the echolocation behavior and foraging strategies of the **frequency modulating (FM)** bat *Natalus tumidirostris*. Our findings reveal this species uses a unique and previously undocumented echolocation strategy in bat literature: a continuous echolocation behavior during the search phase, where 1.2 ms short FM calls are emitted with an average pulse interval of $26 \text{ ms} \pm 6 \text{ ms}$. To our knowledge, no other bat species produces calls uninterruptedly –i.e without arranging their calls in groups– with such a high call emission rate over prolonged periods during the search phase. Furthermore, our research shows that with this echolocation behavior, *N. tumidirostris* is able to detect fluttering insects in highly cluttered environments and so provides for the first time concluding evidence in a behavioral relevant scenario, that a FM bat can detect flutter information. Our recordings also show the calls of *N. tumidirostris* have very low source levels, as well as very high frequency and large bandwidth (the main harmonic sweeps downwards between 152 kHz and 105 kHz). From these call characteristics, as well as adaptations in wing morphology that optimize slow and maneuverable flight, we conclude that *N. tumidirostris*, and likely the entire Natalidae family, are exclusively narrow-space foragers ([Denzinger and Schnitzler \(2013\)](#), [Denzinger et al. \(2018\)](#)). This classification is essential to understand the species' foraging strategies in cluttered spaces, where discerning faint prey echoes from louder clutter echoes presents a significant challenge for echolocating bats.

We know from the literature that bats searching actively with echolocation for prey in narrow space need to be able to discern prey specific signatures in a train of returning echoes and are constantly faced with forward and backward masking effects (see Section 1.2; [Denzinger et al. \(2018\)](#)). Therefore, bats belonging to this narrow space bat

guilds use characteristic foraging strategies and have particular echolocation behaviors to overcome such masking effects. One of the most specialized methods employed by narrow space foraging bats is insect flutter detection, which until now had only been attributed to bats producing long **constant frequency (CF)** calls (see Sections 1.4 & 1.5 and Schnitzler and Denzinger (2011) for review). To our knowledge, this is the first study that provides conclusive evidence under a behaviorally relevant scenario that a FM bat is able to detect flutter information from the insects it hunts. One of our central findings in Chapter 3 indicates that *N. tumidirostris* showed an exclusive preference towards fluttering moths over moths that were not fluttering, something that clearly emphasizes such ability (see Figs. 3.3 and 3.4). Furthermore, we also validated that this species was able to spot moths as close as 10 cm from dense clutter only if they were fluttering (see Section 3.4.2). When hunting for prey, *N. tumidirostris* switches its echolocation behavior from search phase to an inspection phase lasting 0.6 s on average (range: 0.2 s - 2.5 s), in which it reduces the pulse intervals to an average of 18 ms. We also evidenced a switch into the inspection phase when we presented some individuals with a rotating blade while they were hunting (see Chapter 2). All these facts combined confirm that the FM bat *N. tumidirostris* can detect flutter motion from insects.

This dissertation also discusses the possible underlying mechanisms by which *N. tumidirostris* could detect and analyze insect flutter information. We conclude that a single returning FM echo from a fluttering insect can carry flutter information in two distinct forms. First, there is a significant and sudden increase in amplitude contained in an acoustic glint echo compared to previous echoes. Such an increase is often observed within a dominant frequency band at either the beginning or end of the echo (Schnitzler and Denzinger (2011); Kober (1988), Kober and Schnitzler (1990)). Second, the spectral characteristics of a glint echo change due to the interaction between two types of echoes: a Doppler-shifted echo from the insect's moving wings and a non-Doppler-shifted echo from the 'stationary' body of the insect. This interaction creates an interference pattern that results in a complex echo containing conspicuous frequency notches, which the bat could easily identify (Sum and Menne (1988); Mogdans and Schnitzler (1990); Schmidt (1988); Simmons *et al.* (1974); Simmons *et al.* (1990); for more details, see Section 3.5).

Following the detection of potential flutter in form of a glint echo, we conclude *N. tumidirostris* uses its inspection phase to further evaluate and characterize its prey, for example by evaluating the insect's flutter frequency. Our Monte Carlo simulations show

that the higher the flutter frequency of the insect, the higher the glint hit rate, thus suggesting *N. tumidirostris* could use a simple integrator mechanism that counts the number of detected glints per unit of time to estimate the insect's flutter frequency (see Fig. 3.8). However, we believe Compressive Sensing (CS) may provide a much more interesting and plausible system to estimate insect flutter frequency using FM echolocation. CS is a mathematical framework that reconstructs a signal from far fewer samples than traditional sampling methods require, operating under the assumptions that the signal is sparse, with the information concentrated in a few non-zero coefficients after being transformed to a more suitable basis (Fontaine and Peremans (2011), Candès (2006) & Donoho (2006); for more details, see Section 3.5.1). In the context of *N. tumidirostris*, CS would allow the bats to efficiently and rapidly process echo sequences using a reduced number of calls. Based on the results from Fontaine and Peremans (2011), we estimate that *N. tumidirostris* could potentially estimate insect flutter frequencies at or above its own call emission rate of 56 Hz during the typical 0.6 s inspection phase, where pulse intervals average 18 ms.

However, *N. tumidirostris* not only relies on the flutter detection strategy to successfully hunt insects in cluttered spaces. Our dietary analysis using metabarcoding indicates the presence of non-fluttering prey items such as spiders. Furthermore, we evidenced that a very large percentage of the arthropod sequences found in the feces of *N. tumidirostris* are flies of the Tachinidae family. Tachinid flies are characteristic parasites of other insects' larvae (especially of Lepidoptera), so we conclude from parsimony, that the high amount of Tachinid sequences in our scat samples can only be explained due to secondary predation; meaning that the bats are eating (Lepidoptera-) caterpillars infested with Tachinids larvae. Since neither caterpillars nor spiders can flutter, we hypothesize that *N. tumidirostris* must be using the 'active gleaning' hunting strategy to locate such prey items. This hypothesis is in line with our observations of *N. tumidirostris* while hunting in semi-natural conditions, as it was able to sustain hovering flight while echolocating in search of prey. Finally, this diet analysis also lets us conclude that *N. tumidirostris* is a dedicated specialist of Lepidoptera, both from adult moths, as well as caterpillars.

Overall, this dissertation aimed to shed more light and understanding on the foraging strategies and echolocation behavior of *Natalus tumidirostris*. This species employs a unique method of prey detection in cluttered environments, utilizing continuous echolocation with very faint and short, high frequency FM calls that facilitate both flutter de-

tection and active gleaning. The echolocation behavior of *N. tumidirostris* highlights another example of how evolution shaped the echolocation systems of bats to explore acoustically highly demanding foraging habitats.

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