OLFACTORY TRACKING BEHAVIORS OF BATS

A Dissertation

by

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ABSTRACT

Animals rely upon chemical cues to find critical resources for their survival and fitness, including food, shelter, and mates. Behavioral strategies for tracking odor cues are heavily dependent on differences in locomotor and sensory morphology and physiology, which vary substantially across taxa and environments. One of the biggest challenges to olfactory tracking is speed: chemical cues are inconsistent in intensity and temporal and spatial distribution. Consequently, animals engaged in olfactory searches frequently slow down and incorporate lateral movements to reconstruct the local odor structure. Olfactory tracking behaviors have been investigated in many terrestrial vertebrates, but not in flying mammals (bats). Bats are known to use olfaction for communication and foraging and offer an opportunity to evaluate current hypotheses about interactions between ecology, morphology, and behavior when localizing an odor source. In Chapter 2, I applied a phylogenetic framework to investigate whether bat nasal morphology may enhance or constrain the triangulation of odors during odor tracking. Surprisingly, I found that bats known to use odor during foraging (fruit and nectar feeders) had exceptionally narrow separation of the nostrils compared to other species, reflecting a potential trade-off between stereo-olfaction and nasal echolocation. In Chapter 3, I developed a set of behavioral assays using northern yellow-shouldered fruit bats (*Sturnira parvidens*), a Neotropical fruit bat, to quantify the olfactory search strategies of crawling bats. These experiments demonstrated that bats share some similarities with tracking strategies of terrestrial mammals but differ in their use of head

scanning behavior during olfactory search. Finally, in Chapter 4, I used three-dimensional tracking software to characterize the flight paths of Jamaican fruit-eating bats (*Artibeus jamaicensis*) searching for an attractive odor source in a flight cage. These results revealed that flying bats are unlikely to use large-scale odor structure or plume information to guide them to the source. Instead, bats appeared to use a serial sampling and route-following strategy that integrated olfaction and echolocation to quickly and efficiently find the rewarded odor source. Collectively, these results show that bats displayed cognitive strategies that integrate high speeds and biosonar behaviors to optimize their olfactory searches. Understanding the role of olfactory cues in foraging decisions and search behaviors of bats may have important implications for understanding how bats use the landscape for foraging and navigation.

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Planning, executing, and analyzing the work completed for this dissertation was largely carried out by the author. Dr. Smotherman contributed by discussing experimental plans and results and editing many writing samples.

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1. INTRODUCTION

The search for resources, such as food or mates, is fundamental for organisms to survive and reproduce. Chemical cues are critical for the detection, evaluation, and location of resources for many organisms. While following odor plumes is inherently challenging due to the stochastic movement of odor molecules in the environment, animals have developed a variety of traits related to locomotion, sensory detection, and search strategies to locate odor sources. Most of our understanding of olfactory search in vertebrates is from studies in mice, rats, and other terrestrial mammals. Like many other mammals, bats demonstrably rely on chemical cues and signals for communication and foraging, but detailed studies on the behaviors associated with olfactory foraging are still lacking. As the world's only flying mammals, bats experience olfactory landscapes much differently from terrestrial mammals, while also balancing the respiratory demands of flight, fast speeds, and in many species, echolocation. Despite these challenges, many bat species have been shown to use olfactory cues in flight, but the exact strategies with which bats use to overcome these challenges has not been quantified.

1.1. Olfactory Physiology and Tracking Mechanisms

Odor navigation behaviors have two phases: detection, followed by steering towards an odor source (Cohen, 2019), both of which also involve learning, memory and olfactory-based decision-making strategies. The physiological processing of chemical stimuli by mammals is a complex process, involving two functionally and anatomically distinct olfactory systems: the main olfactory system consisting of the main olfactory

bulb (MOB) and olfactory epithelium (OE), and the vomeronasal system, involving the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB) (Dulac and Torello, 2003). Odor perception is facilitated by binding of odor molecules to olfactory receptors (ORs) embedded in the olfactory epitheliums, which initiate signaling cascades to the brain. ORs constitute the largest gene family in mammals (Nei et al., 2008; Niimura and Nei, 2006), with larger OR repertoires associated with the ability to discriminate between more and structurally related odorants (Meisami, 1989; Saito et al., 2009).

Following initial detection and recognition of an odor source, the simplest strategy to determine the direction of a source takes advantage of the physical properties of chemical stimuli, namely diffusion down a gradient. Animals can localize odors by comparing intensity or time of the chemical stimulus (Gardiner and Atema, 2010; Takasaki et al., 2012). Organisms can direct their orientation towards a stimulus by moving their sensory apparatus or body through the environment. *Caenorhabditis elegans* will slowly adjust their movements and perform small turns towards an odor source (Ino and Yoshida, 2009). Other animals, including *Drosophila* larvae, mice, rats, and dogs will move their head (and thus, sensory receptors) laterally while tracking (Baker et al., 2018; Duistermars and Frye, 2010; Gomez-Marin et al., 2010). This klinotaxis allows sequential comparisons of odor concentrations as the animal moves through the environment and involves both spatial and temporal signal integration (Vickers, 2000). In contrast, tropotaxis can enable olfactory orientation via simultaneous comparisons of multiple sensors (Cohen, 2019). Many insects use their antennae to detect and ascend chemical gradients, often turning or biasing their movements towards

a side with a higher odor concentration (Duistermars et al., 2009; Gaudry et al., 2012; Takasaki et al., 2012).

Laboratory studies have demonstrated the importance of multiple sensor or bilateral odor sampling (stereo-olfaction) across a wide range of taxa, including insects (Duistermars et al., 2009; Louis et al., 2008; Steck et al., 2010), mollusks (Basil et al., 2000), crustaceans (Kraus-Epley and Moore, 2002; Pravin and Reidenbach, 2013), sharks (Gardiner and Atema, 2010; Mathewson and Hodgson, 1972), and mammals. Bilateral odor sampling is necessary for rats to determine which side an odor arrives (Rajan et al., 2006) and switching the input sides reversed the directional tracking in humans (Porter et al., 2005; Porter et al., 2007). Use of multiple or bilateral olfactory organs presents an evolutionary advantage for detecting the spatial distribution of odor plumes, such as by reducing time to locate an odor source (Cohen, 2019; Rajan et al., 2006). Mammals are also still capable of following odor trails when a nostril is blocked, although efficiency in tracking is degraded (Catania, 2013; Khan et al., 2012; Porter et al., 2007).

Morphology may constrain the use of different olfactory tracking mechanisms. Klinotactic comparison requires the ability to move receptors within the environment, such as side to side movement of the head while tracking. For tropotactic mechanisms, wider separation of sensory receptors may facilitate better comparison and subsequent triangulation of odors. Stoddart (1979) observed that taxa with particularly limited post-cranial flexibility had wider nostril separation relative to head width and proposed that this may serve as an adaptation for olfactory tracking. Tropotactic scent trailing is

thought to be improved in reptiles with highly bifurcated tongues (Schwenk, 1994), and the wide cephalofoils of hammerhead sharks enable sampling of a wider area, increasing the probability of encountering an odor molecule (Kajiura et al., 2005).

1.2. Odor Plume Dynamics and Animal Movement

With increased distance from the odor source, odor distribution shifts from a relatively predictable gradient to more complex plumes, consisting of complex filaments of odor eddies, surrounded by odorless space (Baker et al., 2018; Murlis et al., 1992). This intermittency (proportion of time when odor signals are absent) is influenced by wind speed, height, and atmospheric stability, creating highly variable odor environments (Murlis et al., 1992). Habitat characteristics, such as vegetation can also alter the intermittency of odor signals in plumes. For example, the intermittency of odor molecules in a plume is reduced in forested environments, compared to open fields where there are many gaps where no signal is present (Murlis et al., 1992; Murlis et al., 2000).

Important insights into how animals navigate the complexity of odor plumes have emerged from insect studies, particularly moth-pheromone interactions (Baker, 1990; Cardé and Agenor, 1994; Cardé and Willis, 2008; Riffell et al., 2008; Willis and Baker, 1987). Many insects (and other invertebrates) use combined sensory inputs to navigate in odor plumes, coupling mechanosensory cues such as wind velocity and direction with olfactory and visual information (odor-gated optomotor anemotaxis) (Cardé and Willis, 2008; Murlis et al., 1992; Vickers, 2000). When searching for an odor plume, or following loss of odor signal due to intermittency, insects respond with cross-

wind, or zig-zag 'casting' movements (Cardé and Agenor, 1994; Cardé and Willis, 2008; Vickers, 2000), which enhance the likelihood of re-contacting the odor plume (Murlis et al., 1992). Following odor detection, moths respond with upwind 'surges' towards the odor source. Similar 'surge-cast' behaviors are observed in response to turbulent odors in other invertebrates, including free-flying *Drosophila* (Budick and Dickinson, 2006) and mosquitoes (*Aedes aegypti*, Dekker and Cardé, 2011; Geier et al., 1999). Other insects, mainly Diptera, are thought to combine mechanosensory information with olfactory information before initiating flight, termed the "aim-then-shoot" strategy (Cardé and Willis, 2008). Fast-flying insects such as tsetse flies (*Glossina*) take off upwind following detection of a host odor, but don't follow wind and plume adjustments in flight (Griffiths et al., 1995). While this strategy might not be as accurate as other plume following strategies, it may enable organisms to quickly sample a large area, particularly when the host or odor source is tens of meters away (Cardé and Willis, 2008). The presence or extend of these mechanisms in vertebrates is still unclear. Observations of foraging procellariform seabirds have shown cross-wind flight patterns followed by upwind flight in response to food-related odors (Nevitt, 2006; Nevitt et al., 2008) and mammalian carnivores such as polar bears (*Ursus maritimus*) may also use cross-wind movements to facilitate olfactory search (Togunov et al., 2017).

Most studies on mammalian tracking mechanisms have focused on the strategies for scent-trail following, where odors are deposited on the ground or a surface. Odors deposited on substrates can have fundamentally different chemical properties, such as higher molecular weight and lower volatility, which allows persistence in the

environment over a longer period of time (Mollo et al., 2017), particularly when used for a specific purpose, such as ant food location trails (Jackson and Ranieks, 2006). Odors emanating from near the boundary layer, such as those encountered by walking organisms, are also less turbulent and complex compared to plumes in more open air (Baker et al., 2018). Mammals following scent trails display zig-zag casting mechanisms similar to that observed in flying moths (Jinn, 2019; Khan et al., 2012; Porter et al., 2007), usually initiated in response to loss of contact with the odor trail (Khan et al., 2012).

1.3. Integrating Olfaction with Other Behavioral Strategies

Following an odor plume or odor source can be imagined as a problem of when and where to sample an odor, behaviors that animals can modulate depending on the olfactory task. Animals can and will also shift their strategies as the olfactory environment changes, such as distance from the odor source. At farther distances, gradients are shallower or non-existent (particularly in the case of turbulent eddies). Large movements and serial sampling (klinotaxis) in different areas can provide directional information, while at closer distances stereo-olfaction and bilateral comparisons (tropotaxis) may be sufficient to provide directional cues (Catania, 2013). Behaviors such as increased sampling rate (sniffing) and head-scanning can occur simultaneously with these mechanisms. Dogs will increase their sniffing frequency while deciding track direction (Thesen et al., 1993), and rats demonstrate higher sniff rates during tracking than non-tracking (Khan et al., 2012). Humans will also modulate their sniffing behaviors, by either increasing their sniffing rate (Porter et al., 2007) or length

of each sniff (Jinn, 2019). Head-scanning behaviors have been observed in several mammals, including rats, moles, dogs, and humans (Catania, 2013; Khan et al., 2012; Porter et al., 2007). This movement of the nose might improve the efficiency of klinotactic localization by allowing the animal to maintain its general position within an odor plume, while also allowing a longer period to sense and integrate a chemical signal (Dusenbery, 1992).

Even with increases in sampling rate, mammals are still constrained by the speed of olfactory processing (Chittka et al., 2009; Rinberg et al., 2006) and locomotor speed can have a major effect on olfactory cue discovery and localization. Fast moving animals searching for resources can cover more territory but may do so at the expense of olfactory detection. To compensate for increased turbulence or variation in concentrations, animals will typically adjust their locomotor patterns. In more turbulent flows, crustaceans will move slower and walk in a more undulating path in order to increase their chance of detecting the plume (Moore and Atema, 1991; Moore et al., 1991; Weissburg and Zimmer-Faust, 1994). Mammals have been observed using similar strategies. Dogs tracking a scent trail slow down to half their speed when deciding the direction of an odor track (Thesen et al., 1993) and coati foraging for fruit moved in a pause-travel fashion, with increased sampling or sniffing during pauses (Hirsch, 2010).

1.4. Olfactory Foraging Behavior in Bats

Morphological and behavioral studies support the integration of olfactory cues by foraging bats, particularly in fruit and nectar-feeding species. Bats demonstrate a highly diverse OR repertoire compared to other mammals (Hayden et al., 2010) with unique

and convergent patterns differentiating fruit-eating bats from animalivorous bat species (Hayden et al., 2010; Hayden et al., 2014). Fruit-eating bats from Central and South America (Family: Phyllostomidae) have significantly different OR repertoires compared to non-fruit eating relatives, suggesting a strong association between OR diversity and specialization on fruit. Comparative studies of bat brain and nasal anatomy also suggest a strong link between dietary ecology and olfactory capabilities. Frugivorous bats (both echolocating and non-echolocating species) have more foramina in the cribriform plate, suggesting more neuron connections associated with olfaction (Bhatnagar and Kallen, 1974a). The size of the olfactory bulb was also larger in bats that use olfaction for foraging (fruit and nectar feeding bats) (Bhatnagar and Kallen, 1974a; Bhatnagar and Kallen, 1974b) with the main olfactory bulb most developed in non-echolocating fruit bats (Family: Pteropodidae) (Hutcheon et al., 2002). Anatomical variation in the nasal cavities also suggest reliance on olfaction by plant-visiting species; the fruit-eating bat *Artibeus jamaicensis* has more complex ethmoturbinals with more surface area of olfactory epithelium, and nearly twice as many olfactory receptors as the insect-eating *Myotis lucifugus* (Bhatnagar and Kallen, 1974a).

Early observations of the strong odors of bat pollinated flowers and dispersed fruits suggested a possible role of olfactory cues for foraging (Van Der Pijl, 1957). In the non-echolocating fruit bats (Family: Pteropodidae), olfactory cues play a role in detection and discrimination of fruit, mostly at short distances (Acharya et al., 1998; Luft et al., 2003; Raghuram et al., 2009; Tang et al., 2007). Behavioral studies also suggest that bats are able to assess ripeness and quality of fruit based on odor cues

(Hodgkison et al., 2007; Luft et al., 2003; Sánchez et al., 2006). Ripe fruit cues also play an important role in foraging discrimination by short-nosed fruit bats (*Cynopterus brachyotis*), both with and without visual cues (Hodgkison et al., 2007). Bats also appear to discriminate between variations in volatile compounds, with *C. brachyotis* demonstrating higher reaction rates to natural fruit odors compared to synthetic combinations (Hodgkison et al., 2007) and Egyptian fruit bats (*Rousettus aegypticus*) used volatiles produced during fermentation (such as ethanol) to avoid over-ripe and low-quality fruit, although this was only demonstrated with unnaturally high levels of ethanol in behavioral experiments (Sánchez et al., 2006).

Fruit and nectar-feeding bats within the Phyllostomidae also rely on olfactory cues while foraging, combined with navigations via echolocation (Korine and Kalko, 2005; Leiser-Miller et al., 2020; Parolin et al., 2015; Thies et al., 1998). Mist nets baited with banana odors in Costa Rica caught more fruit-eating bats of some subfamilies, but not others, indicating that some frugivorous bats respond to odors while foraging (Rieger and Jakob, 1988). In environments with high background clutter (such as forest understory or clustered fruits on a leafy branch), echolocation may be inefficient for detecting specific objects (Schnitzler et al., 2003; Thies et al., 1998). Odors can serve as general location and detection cues for ripe fruit in these environments and are often necessary to stimulate feeding behaviors (Kalko and Condon, 1998; Korine and Kalko, 2005; Thies et al., 1998). Bat species also demonstrate strong preferences for fruit odors of different species, for example with *Artibeus* species generally showing strong preferences for *Ficus* figs (Bianconi et al., 2007; Parolin et al., 2015), and species within

the genus *Carollia* discriminating between volatiles of different *Piper* species (Leiser-Miller et al., 2020; Mikich et al., 2003).

Neotropical fruit-eating bats, such as *Carollia perspicillata*, are highly sensitive to many fruit-typical odor compounds, including alcohols, carbon acids, and esters (Laska, 1990a). *Carollia* is also able to discriminate odor quality and quantities, a first step in being able to recognize an odor gradient (Laska, 1990b). Other Neotropical bat species such as the fig specialists in the genus *Artibeus* demonstrate positive relationships with monoterpenes, which are released during early stages of ripeness (Parolin et al., 2019) and dominate the odors of bat-dispersed figs across the world (Hodgkison et al., 2013). Interestingly, the Neotropical fruit-eating bat *A. jamaicensis* demonstrated a preference for monoterpene scented figs from both Neo- and Paleotropical regions, while the Paleotropical fruit bat *C. brachyotis* only responded to odors from bat figs in their native region (Hodgkison et al., 2013).

Odor cues play a large role in attracting nectar feeding bats to flowers. Many species of Neotropical bat-pollinated plants have strong, unpleasant odors due to production of sulfur compounds (Bestmann et al., 1997), which have been demonstrated to be strong attractants for flower-visiting bats (von Helversen et al., 2000). Chiropterophilic flowers in Central and South America display traits that facilitate detection via echolocation, such as distinct concave shape that directs echolocation pulses to the center (von Helversen and von Helversen, 1999) or cauliflory and flagelliflory (where flowers are positioned away from the trunk or crown of foliage) (Diniz et al., 2019; Muchhala, 2006). While bats can respond to either echolocation or

olfactory cues, they often react faster and display preferences for combined cues (Gonzalez-Terrazas et al., 2016b). Interestingly, sulfur compounds are rarely found in African and Asian bat-pollinated flowers (Pettersson et al., 2004) and did not attract the flower-visiting dawn bat (*Eonycteris spelea*) in Thailand (Carter and Stewart, 2015). Compared to nectar feeding bats in the Americas, the use of odors by Paleotropical flower bats has not been tested in many behavioral experiments.

Evidence for use of olfactory cues during foraging in non-phytophagous species is limited. Vampire bats (*Desmodus rotundus*) have a well-developed sense of smell (Greenhall et al., 1983; Laska, 1990a) and have been shown to prefer blood meals associated with scented cues (Bahlman and Kelt, 2007). However, it is still unclear at what spatial scale they use these odor cues. Insectivorous bats may rely partially on olfactory cues for prey discrimination, as a way to avoid unpalatable species (Kolb, 1961), and several species may be able to locate hidden insects by smell within a few centimeters range (Stoddart, 1980). However, in experiments on insectivorous species in the subfamilies Kerivoulinae and Murininae, bats still caught, or attempted to catch, insect dummies that lacked olfactory or gustatory cues, suggesting these senses do not play a strong role in foraging by insectivorous bats (Schmieder et al., 2012). Acoustic information can provide more accurate localization, particularly for moving objects such as flying insects, while olfactory location cues are more intermittent and delayed.

There is ample evidence that like other mammals, many bats can and do use olfactory cues and signals for foraging, but the mechanisms and behaviors by which they follow odors plumes is still unclear. Compared to most terrestrial mammals, bats are

searching and foraging at higher speeds, while balancing the physical and respiratory demands of maintaining flight and in many species, supporting echolocation. It is unclear how some of the strategies that terrestrial mammals use to overcome challenges associated with speed (such as slowing down, increasing sampling rate, or head-scanning) translate to natural bat foraging behavior. While bats are observed to increase their sniff rates in response to olfactory stimuli, this has only been rigorously tested in stationary bats, who were not also actively echolocating (Laska, 1990a). Bats can also use side-to-side head movements to direct sonar pulses during flight (Seibert et al., 2013) and so may also be able to take advantage of this strategy when navigating in an odor plume. Echolocation in bats is linked to the respiratory and wing-beat cycle, with echolocation pulses generally emitted during expiration (Falk et al., 2015; Suthers et al., 1972) and it is unclear how the demands of echolocation, respiration, and sniffing interact in flying bats. To address some of these gaps, the main goal of this dissertation is to examine the interactions between ecology and olfactory tracking behaviors in foraging bats.

In Chapter 2, I compared the external nasal morphology of bats that differ in their use of olfactory cues while foraging, to test the hypothesis that morphological adaptations associated with the nose may help support olfactory tracking in flight. Taking advantage of the diversity of nostril morphology and ecology in bats, I used museum specimens from 40 bat species and a phylogenetic comparative framework to establish if there is a link between nostril morphology and olfactory perception or tracking. If nostril separation plays a role in olfactory-guided behavior, then bats that use

olfactory cues while foraging (e.g., frugivores and nectarivores) are predicted to have wider nostrils relative to head width than bats that feed on insects (less reliant on olfactory foraging cues).

To quantify the olfactory search behaviors of bat localizing food-based odors, I developed and validated a set of behavioral assays, focusing on two frugivorous species in the family Phyllostomidae: the northern yellow shouldered bat (*Sturnira parvidens*) and the Jamaican fruit-eating bat (*Artibeus jamaicensis*). Fruit-eating bats in this family have been previously shown to rely on olfactory cues while foraging (e.g. Korine and Kalko, 2005; Mikich et al., 2003; Parolin et al., 2015; Thies et al., 1998). Both of these species are abundant and common through their range (Reid 2009), and robust to temporary captivity for behavioral experiments (Hodgkison et al., 2007; Mikich et al., 2003; Parolin et al., 2015).

If bats are not able to compensate morphologically or physiologically to the demands of flight and echolocation, they may instead supplement their use of sensory information with memory-guided navigation such as cognitive maps (Baker et al., 2018; Geva-Sagiv et al., 2015). Mice and rats are able to localize the source of an odor plume without relying on casting behaviors, and under certain circumstances, other strategies such as serial sampling may be faster and more robust to atmospheric changes than plume following (Bhattacharyya and Bhalla, 2015; Gire et al., 2016). Learning may also have an effect on olfactory localization behaviors, with mice first using sensory cues to navigate in an odor plume but switching to a more stereotyped serial search strategy after gaining familiarity with the task (Gire et al., 2016). Bats have excellent spatial

working memories (Toelch et al., 2008; Winter and Stich, 2005) and may be more likely to rely on spatial memory rather than sensory cues when foraging (Carter et al., 2010; Fleming et al., 1977). In primates, the use of odors for long distance detection appears to be limited, and olfactory cues may instead be important for food evaluation and selection (Dominy, 2004; Laska et al., 2007; Rushmore et al., 2012). It may also be that bats are able to follow odor gradients while crawling, but under the more challenging olfactory conditions of flight instead rely on other search mechanisms.

In Chapter 3, I developed and validated a behavioral assay for olfactory tracking in crawling individuals, using the northern yellow-shouldered bat (*Sturnira parvidens*). The northern yellow-shouldered bat is a small frugivore (13 – 18 g) common to much of Central America and frequently captured in ground nets in Belize, where this study took place (Reid 2009, Fenton et al. 2001). Bats were trained and presented with a choice between a control and odor-infused reward to test how well bats can locate food odors and examine the effect of odor concentration on performance. At this restricted spatial scale, bats were predicted to use odor concentration gradients (klinotaxis) to evaluate their position relative to the odor source and are expected to adjust their search behaviors as the difficulty of the task increase (decreasing odor concentration). In Chapter 4, I adapt this behavioral assay to characterize the olfactory tracking behaviors of bats in flight, using three-dimensional flight reconstruction. This study focused on another frugivorous leaf-nosed bat, the Jamaican fruit-eating bat (*Artibeus jamaicensis*), which is common in Gamboa, Panama, where flying assays were conducted. Flying animals are exposed to a much more turbulent and variable olfactory environment, which may result

in a shift in behavioral search strategies. Although bats appear able to discriminate odors in flight, it is unknown if they are able to use odor cues while flying to follow a plume to its source. Given constraints of odor tracking while in flight (speed, echolocation, and respiration), bats may be able to follow odor plumes, suggesting extraordinary odor sensitivity or neuro-processing. Alternatively, bats may instead rely on memory or spatial strategies for locating a food reward, using odors as a cue for final discrimination or choice.

With over 1,400 described species, bats make up approximately 20% of all mammals (Simmons and Cirranello, 2020. In Chapter 5, I synthesize the results of these studies and discuss how morphological variation may affect olfactory tracking, the possible constraints imposed upon olfaction by echolocation, and the sensory and cognitive strategies bats use to overcome these challenges. Although this dissertation focuses on olfactory behaviors associated with foraging, I also discuss how morphology and behavioral strategies for olfactory foraging may relate to use of olfactory cues in bat communication in Chapter 5. This dissertation is the first concerted effort to characterize the behavioral strategies employed by bats to perform olfactory searches and expands our understanding of strategies associated with olfactory search by adding information about this major group of mammals, and their unusual constraints (flight and echolocation).

2. ROLE OF ECOLOGY IN SHAPING EXTERNAL NASAL MORPHOLOGY IN BATS AND IMPLICATIONS FOR OLFACTORY TRACKING[*]

2.1. Introduction

Animals rely on chemical signals to detect, identify, discriminate, and localize the resources critical for their survival and fitness, including food, shelter, and mates. Tracking an odor to its source (localization) is a complex task, integrating the internal characteristics of an organism (such as nasal anatomy, receptor physiology, central sensory integration circuits, locomotion patterns, etc.) with the physical characteristics of the chemical odor and the surrounding environment (Svensson et al., 2014). Odors move through the environment in complex, discontinuous and variable odor plumes, presenting a complex environment where animals must rely on various algorithms or strategies in which to extract and use odor information from the environment.

Animals also display diverse behavioral responses and strategies for following an odor trail to its source that vary with habitat, size, and locomotor speeds. Olfactory klinotaxis (or true gradient search) is movement through an olfactory gradient with successive sampling at different locations (Dusenbery, 1992). To be effective, this strategy requires close proximity to the odor source, since at farther distances turbulence and advection begin to create patchier distributions of odor concentrations. Olfactory

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[*] Adapted with permission under open access license "CC-BY" from PLOS ONE. Brokaw and Smotherman 2020. Role of ecology in shaping external nasal morphology in bats and implications for olfactory tracking. *15*(1), e0226689.

tropotaxis is the ability to simultaneously compare odor inputs among multiple receptors, such as antennae or nostrils (Dusenbery, 1992). Animals can use tropotactic mechanisms to orient towards an odor based on concentration gradient (Takasaki et al., 2012) or time of odor arrival (Gardiner and Atema, 2010) Bilateral processing of odors (stereo-olfaction) is crucial in the olfactory localization behavior of a wide range of taxa, including insects (Duistermars et al., 2009; Steck et al., 2010), mollusks (Basil et al., 2000; Wyeth, 2019), crustaceans (Kraus-Epley and Moore, 2002), fish (Gardiner and Atema, 2010) and mammals (Catania, 2013; Khan et al., 2012; Porter et al., 2007; Rajan et al., 2006). Bilateral odor sampling is necessary for rats to determine which side an odor arrives (Rajan et al., 2006) and a rat's ability to follow a scent trail is degraded when a single nostril is blocked (Khan et al., 2012). Stereo-olfaction has also been shown to play a role in odor localization and tracking in moles (Catania, 2013) and humans (Porter et al., 2007).

Most previous comparative studies on animal olfactory capabilities have focused on measures reflecting olfactory sensitivity. For example, neuroanatomy and skull morphology have been shown to be strongly correlated with olfactory receptor gene repertoires in mammals, thus serving as a viable metric for olfactory capacity across species (Bird et al., 2018). Conditioning paradigms and behavioural assays have been used to evaluate sensitivity to different chemical compounds in some mammals, mainly in mice (Can Güven and Laska, 2012; Schellinck et al., 2001) and primates (Eliasson et al., 2015; Hübener and Laska, 2001; Rushmore et al., 2012). However, measures of olfactory sensitivity and discrimination cannot tell us very much about the behavior

animals use to track an odor to its source. External nasal morphology may give insight into what behavioral mechanisms animals are using to locate odor sources, particularly anterior nare placement and spatial olfactory information. In this study, we used phylogenetic comparative methods to test the hypothesis that external nasal morphology should vary with potential olfactory tracking capabilities.

With over 1,400 species and considerable variation in morphology and ecology, bats offer many opportunities for investigating ecological and evolutionary questions in a comparative framework. Olfaction is used during foraging by many bat species, particularly fruit and nectar feeding bats (Gonzalez-Terrazas et al., 2016a; Korine and Kalko, 2005; Rieger and Jakob, 1988; Sánchez et al., 2006; von Helversen et al., 2000). Seba's short-tailed fruit bats (*Carollia perspicillata*) display enhanced sensitivity to fruit-typical odor compounds, and can discriminate odor quality and quantities, a first step in being able to recognize and follow a concentration gradient (Laska, 1990a; Laska, 1990b). Frugivorous bat species have enhanced olfactory acuity and increased reliance on olfactory cues (Bhatnagar and Kallen, 1974b; Hutcheon et al., 2002). Bats are hypothesized to use olfactory cues for initial detection and discrimination at long distances, followed by echolocation for exact localization at close distances (Korine and Kalko, 2005). In environments with high background clutter (such as forest understory), echolocation may be an inefficient mechanism for detecting objects even at close ranges, making olfactory cues all the more important for detecting and localizing food resources (Muchhala and Serrano, 2015). Bats also display a surprising diversity of nostril size,

shapes, and orientation (Figure 2.1), the drivers of which are still not well understood (but see Pedersen and Müller, 2013).

Figure 2.1. Examples of nose shape, nostril shape, and nostril positioning of species included in this study. Top, left to right: Black mastiff bat (*Molossus rufus*), Striped hairy-nosed bat (*Gardnerycteris (Mimon) crenulatum),* Hairy big-eyed bat (*Chiroderma villosum*). Bottom, left to right: Hoary bat (*Lasiurus cinereus),* Common vampire bat (*Desmodus rotundus*), Yuma myotis (*Myotis yumanensis*). Photographs by A.F. Brokaw.

In this study we evaluate if there is a relationship between external nasal morphology and foraging ecology among bats. As flying vertebrates, bats face a more complex fluid environment for olfactory tracking than terrestrial animals, while also having less behavioral flexibility to compensate for these challenges (such as ability to slow down or pause while sampling). Stoddart (1979) proposed that wider separation between receptors (e.g., external nares in vertebrate) may enhance olfactory tracking

navigation by increasing the effective sampling area of an organism or by increasing the ability to detect and resolve differences in odor concentration (Kajiura et al., 2005; Takasaki et al., 2012) or arrival timing (Gardiner and Atema, 2010). Based on these hypotheses, we predicted that bat species known to use odor while foraging would have broader separation of the nostrils compared to species that predominantly rely upon acoustic cues (i.e,. insectivorous bats). Foraging habitat and flight capabilities may also exert selective pressure on olfactory tracking and nasal morphology, by changing the relative importance of sensory inputs (i.e., odor and echolocation in cluttered habitat; Muchhala and Serrano, 2015)), or ability to move or change speed within the odor plume (i.e., flight maneuverability). Bats that forage in open environments or that have limited maneuverability while foraging (high aspect ratios, fast flight speeds) would be more constrained by their sampling ability and would be predicted to have wider nostrils than more maneuverable species.

2.2. Materials and Methods

2.2.1. Morphological Data

We measured eight external nasal and body measurements on 40 New World bat species from four different families (Molossidae, Mormoopidae, Phyllostomidae, and Vespertilionidae) (Figure 2.2, Table 2.1). Measurements were taken from a total of 328 fluid-preserved specimens, located at the Biodiversity Research and Teaching Collections at Texas A&M University (College Station, Texas, USA). We recorded measurements from between two and 11 individuals per species (with an average of eight individuals measured per species). All linear external measurements were taken to

the nearest 0.01 millimeter using digital calipers. All forearm measurements were taken from the right wing, where possible. The inner nostril width ratio (INWR) was calculated by dividing the cranial width by the inner nostril width, after Stoddart (Stoddart, 1979). To reduce measurement error, all samples were measured by the same person (A.F.B.). For each species, measurements from each character were examined for outliers and these specimens were excluded from further analysis to further reduce measurement bias. Only specimens that were intact with no bone or organ removal, and soft tissues (especially nose-leaves) in a natural position with no severe angles or deformation were selected for measurements. Alcohol and other preservation methods for animal specimens can have strong effects on measurements such as body mass (Vervust et al., 2009), and metadata for fluid-preserved specimens are rarely consistently recorded or digitized. Therefore, average body mass for each species was obtained from the PanTHERIA database (Jones et al., 2009), with the exception of *Molossus rufus* (Reid, 2009), *Myotis nigricans* (Wilson and LaVal, 1974) and *Lonchophylla handleyi* (Solari et al., 1999).

To try to estimate potential shrinkage effects of preserved specimens, we collected morphometric data from live bats (n = 4 – 6) each for 10 species included in this analysis. Live bats were captured using mist-nets in Lamanai, Orange Walk District, Belize (17.75117 N, −88.65446 W) and were released following processing. All methods were approved by the Belize Forest Department (permit number FD/WL/1/19(10) to A. Brokaw) and the Texas A&M University Institutional Animal Care and Use Committee (AUP 2017-0139). We calculated the percent change between live animals and

preserved specimens for each morphological character (Figure 2.2, Table 2.1), for each species. Specimens had an average loss of 2.67% across all morphological characters. Forearm had a lower average percent shrinkage than all other measurements (mean = -1.76%, one-way ANOVA, $F = 3.284$, $P = 0.007$, Appendix A). There was no difference in percent shrinkage across morphological variables when compared between species (one-way ANOVA, $F = 0.504$, $P = 0.868$). While specimens did show shrinkage compared to live animals, the difference was consistent across measurements and species, so we feel confident that any differences observed in our dataset reflect the variation in living organisms.

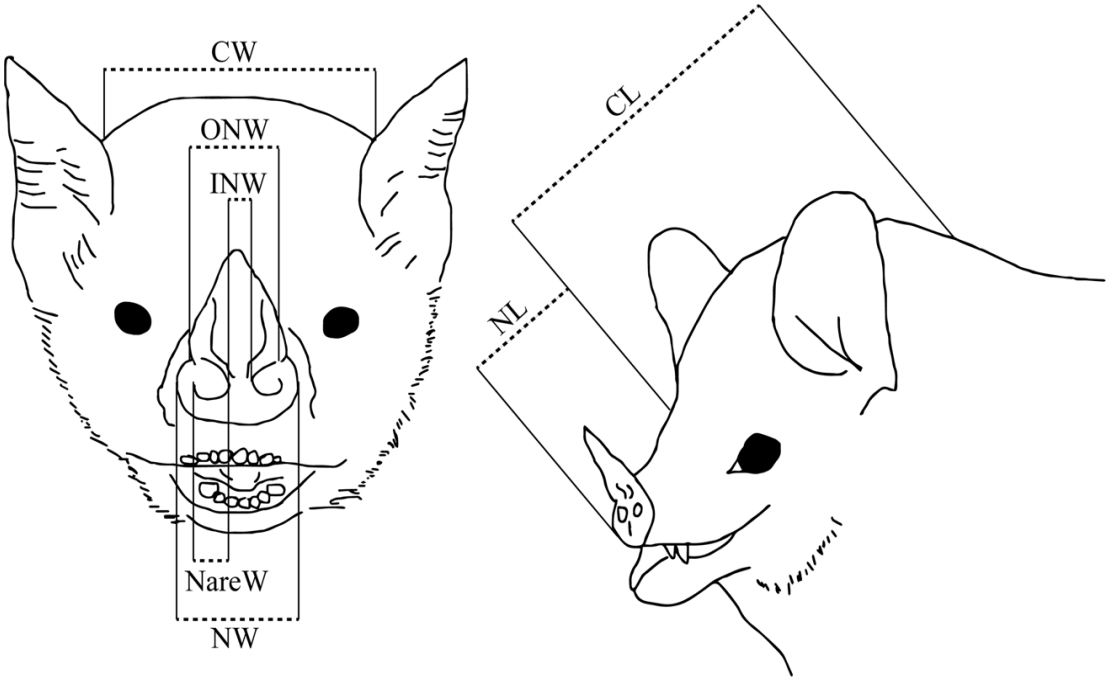

Figure 2.2. Facial morphological characters examined from alcohol-preserved museum specimens. See Table 2.1 for abbreviations and descriptions.

Previous studies on olfactory search strategies in mammals have shown the importance of stereo-olfaction and tropotaxis in rats (Khan et al., 2012; Rajan et al.,

2006) and mice (Liu et al., 2020). While not included in the statistical analyses, we also collected morphometric data from fluid-preserved museum specimens of rats (*Rattus rattus*) and mice (*Mus musculus*) as a reference to compare to bat values.

Table 2.1. Description of morphological characters. All characters were measured in millimeters (mm).

Character	Description
INW	*inner nostril width*: minimum distance between the inner edges of the external nares
ONW	*outer nostril width*: minimum distance between the outer edges of the external nares
NL	*nose length*: distance from tip of nose to midpoint between the eyes
NW	*nose width*: maximum distance between the outer part of the nose/rhinarium
CL	*cranial length*: distance from base of occipital bone to midpoint between the eyes
CW	*cranial width*: maximum distance of the head measured immediately anterior to the ears
INWR	*inner nostril width ratio*: ratio of the cranial width to the inner nostril width
NareW	*nare width*: average maximum distance across the external nares

2.2.2. Ecological Data

Using published data, we classified each species into categories that reflect their foraging and flight behavior (Appendix A). Species were assigned to one of five dietary categories. Bats whose diets are known to contain large proportions of both plant and animal material were classified as omnivores. Foraging habitat and mode were assigned from the literature, modified from the classification scheme presented by Denzinger and Schnitzler (2013) to serve as a proxy for overall flight abilities. Habitat and vegetation complexity can have an effect on the distribution of odors in the environment, thus

influencing movement of odor plumes (Murlis et al., 2000). We define three types of foraging habitat, based on the amount of environmental clutter: open space, edge space and narrow space. Foraging mode refers to method of prey acquisition: aerial (acquire prey from air) or gleaning (taking food from off a surface). For a subset of species, we also recorded average flight speed (21 species), wing loading (25 species), and aspect ratio (35 species) from the existing literature to quantitatively evaluate the relationship between flight ability and nose morphology. External nasal morphology is also mechanically linked to echolocation in species that emit echolocation pulses through their nostrils (Hartley and Suthers, 1987), so each species was classified as either a nasal or oral emitting echolocator. Bats were classified as either migratory (undergoing long-distance seasonal migration) or non-migratory, based on existing literature.

2.2.3. Statistical Analysis

Closely related species tend to resemble one another, resulting in lack of statistical independence and pseudo-replication (Felsenstein, 1985). To account for shared ancestry, we performed all analyses within a phylogenetic context. We based our phylogeny on the one used by Shi and Rabosky (2015), a time-calibrated, maximum-likelihood phylogeny of extant bats based on a 29-locus genetic super matrix, which we then pruned to include only the 40 species in this study (Figure 2.3). We performed all analyses using the ‘ape, ‘caper’, ‘geiger’ and ‘phytools’ packages in R, version 3.5.0 (Harmon et al., 2008; Orme et al., 2018; Paradis and Schliep, 2019; Revell, 2012).

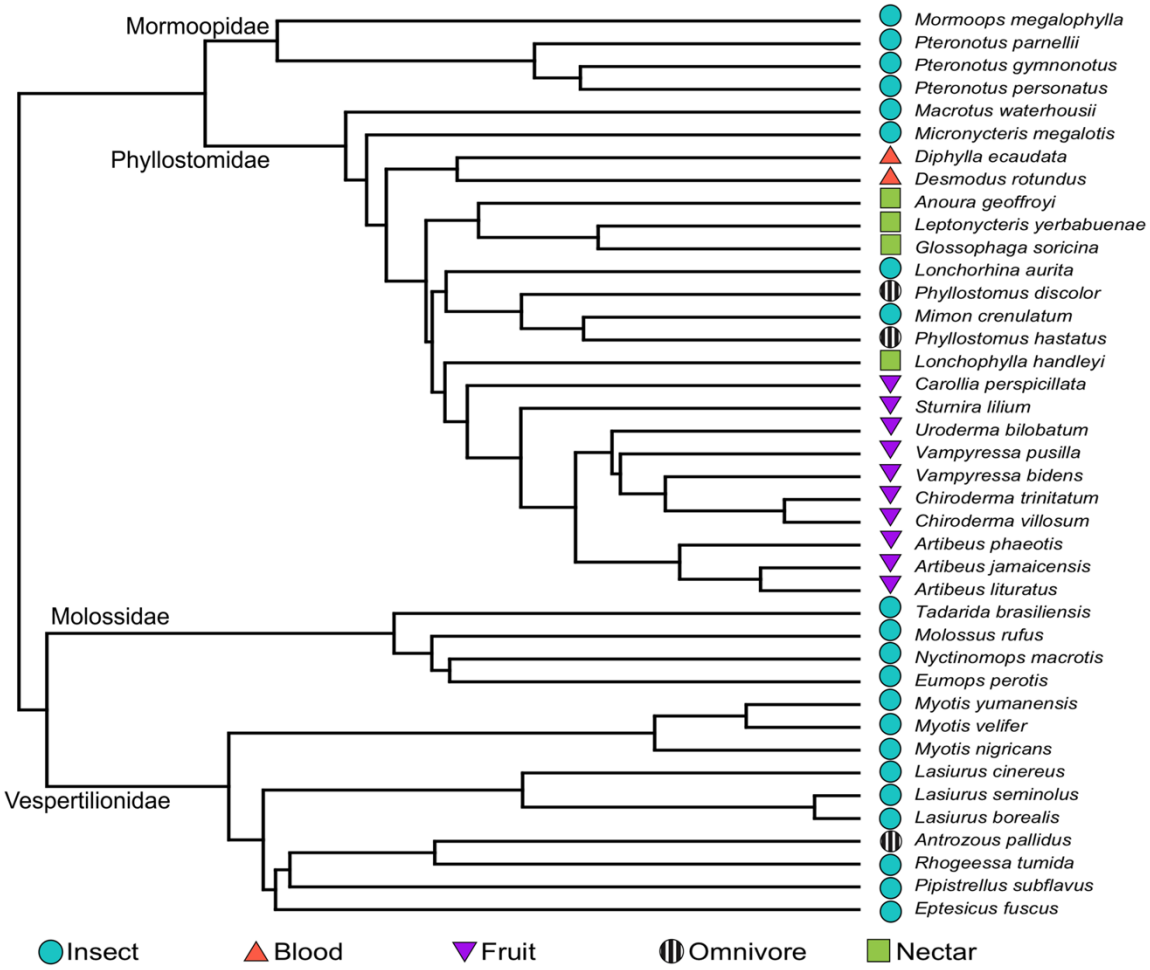

Figure 2.3. Phylogenetic tree used for phylogenetic comparative methods. Tree is derived from the species-level phylogeny of Shi and Rabosky (2015) and pruned to include only species examined in this study. Symbols represent diet categories.

Prior to analysis, all morphological variables were log-transformed to meet the assumptions of normality and the data was inspected for outliers. The phylogenetic signal for morphological variables were estimated using Pagel's λ and Blomberg's *K* (*phylosig* function, *phytools*; Revell, 2012). Pagel's λ (Pagel, 1999) is an estimate of the correlation between species relative to the correlation expected under Brownian evolution, on a scale between 0 (no correlation between species, equal to a star

phylogeny) and 1 (correlation between species equal to Brownian expectation). Blomberg's K is a measure of the partitioning of variance among clades. $K < 1$ indicates that closely related species are less similar to each other than expected by Brownian motion, while $K > 1$ means that closely related species are more similar to each other than expected (Blomberg et al., 2003).

We were interested in how morphology of the nose and head, particularly width of the nostrils, is influenced by diet and other ecological variables. However, nearly all measured morphological variables were highly correlated with each other, even when accounting for phylogenetic non-independence (PGLS regressions, $P < 0.05$). We performed a phylogenetically informed principal component analysis (pPCA) on the mean morphological variables to explore the co-variation between variables and obtain independent axes of variation, using *phytools* (Revell, 2012). Cranial width and inner nostril width were excluded from the pPCA because they were used to calculate the inner nostril width ratio. Relationship between the head and nose morphology and ecological variables were tested using phylogenetic generalized least squares (PGLS) regressions based on species' scores obtained from the pPCA (*pgls* function in the *caper* package, Orme et al., 2018). The optimal value of lambda was estimated using maximum likelihood during calculation of the PGLS. We regressed each principal component (PC) separately on the ecological variables. To control for differences in body size across species, we used body mass and average forearm length as covariates in the models, modelled separately. Size measures were used as covariates instead of size-corrected residuals because the morphological variables were collinear, and the use of

residuals in model fitting can result in biases in phylogenetic data (Freckleton, 2009). Model selection was performed through comparisons using Akaike Information Criteria corrected for small samples sizes (AICc, Burnham and Anderson, 2004)).

Principal component values can be difficult to interpret in a biological context, so we also compared the inner nostril width relative to head (INWR) of bats across different ecologies. This is the measure most directly related to separation of air streams between the two nares and is therefore hypothesized to be the most relevant to potential olfactory tracking mechanisms, such as tropotaxis (Stoddart, 1979). We used phylogenetic ANOVAS to test for differences across diet, foraging habitat, and echolocation mode, and conducted *post-hoc* comparisons of means for the statistically significant tests, adjusted using the Holm-Bonferroni correction to account for multiple testing.

Roughly half of the species in this study belong to one family (Phyllostomidae, 22 species), all of whom are primarily nasal echolocators. Phyllostomidae is a highly diverse group of bat species with a wide range of ecological variation in morphology, diet and ecology (Freeman, 2000). We applied the above analyses just within this family, to see if and how these relationships change across and within the phylogeny.

2.3. Results

2.3.1. Phylogenetic Signal

We found weak signal (less than expected under Brownian motion) for both average mass and forearm length (Blomberg's $K < 0.6$, $P > 0.05$; Pagel's $\lambda < 0.5$, $P > 0.05$). Cranial width also showed a weak phylogenetic signal, which was further reduced

when *Eumops perotis* was excluded from the analysis. Measurements of nose morphology (INW, ONW, NL, NW, and INWR) showed strong phylogenetic signal (Blomberg's $K > 0.7$, $P < 0.05$; Pagel's $\lambda > 0.8$, $P < 0.05$), implying that related species have more similar nose morphology than expected under Brownian motion of evolution (Figure 2.4). When looking only at the Phyllostomidae, there was weak phylogenetic signal for nearly all morphological variables measured in this study, further suggesting a non-random pattern in morphology across the entire dataset (Appendix A).

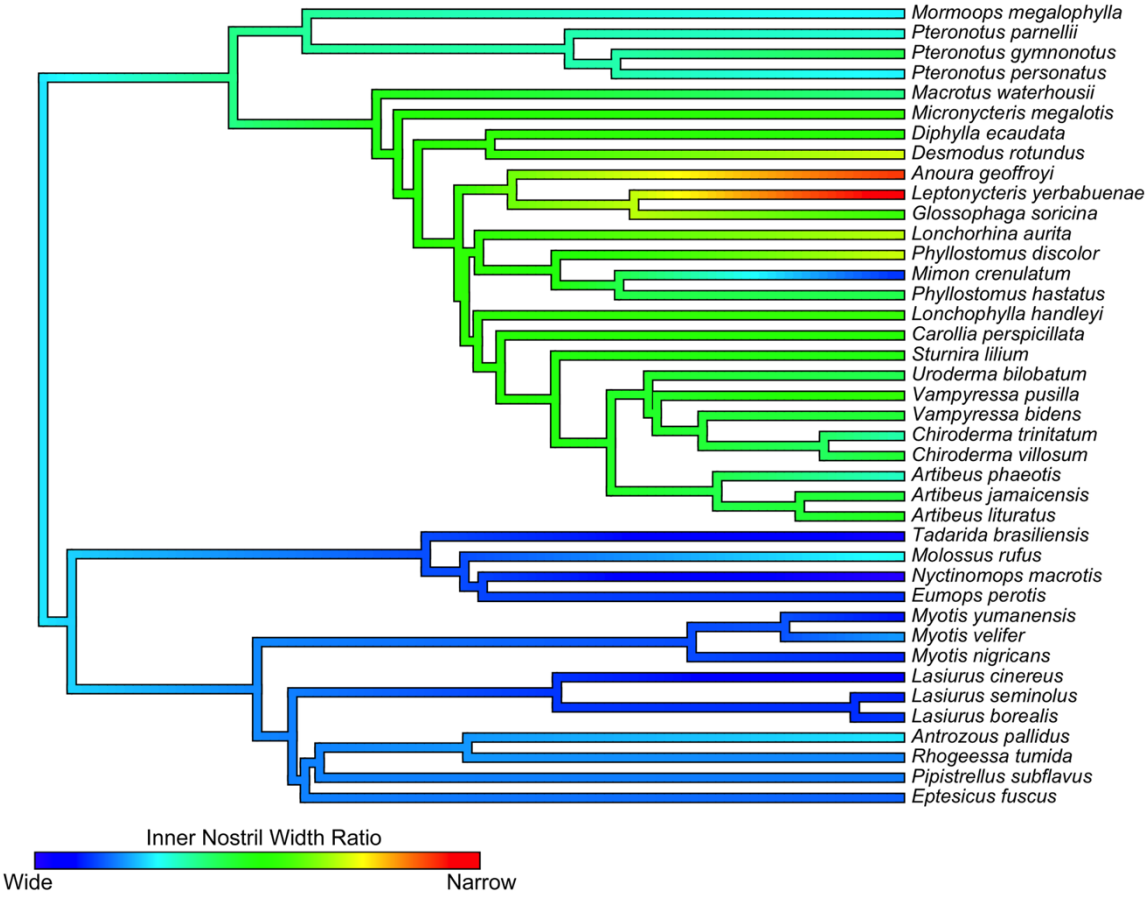

Figure 2.4. Ancestral character estimation of inner nostril width ratio (INWR). Red colors indicate species with narrow nostrils relative to head width, while dark blue indicates species with relatively wide nostrils. Illustration made in R (*contMap* function, package *phytools;* Revell 2012).

2.3.2. Variation in Nose and Cranial Morphology

Morphometric analysis reveals substantial variation in the nose and head morphology among bats. A summary of morphological measurements for species included in this study is given in Appendix A. Linear inner nostril separation distances from ranged from less than 1 mm (0.77 mm in *Anoura geoffroyi*) to 5.6 mm in *Eumops perotis*, compared to an average 2.01 mm from rodent specimens. The greatest relative separation (INWR) in bats was found in *Nyctinomops macrotis* (3.2) and smallest relative separation in the nectar-feeding species *Leptonycteris yerbabuenae* (14.88). A phylogenetic principal component analysis (pPCA) on the mean morphological variables yielded three component axes that jointly explain 93.8% of the variation (Appendix A). The first component (PC1, 67.3% of variance) was strongly affected by ONW (loading = -0.911), NW (-0.897), CL (-0.906), and NareW (-0.915), indicating an overall measure of face and nose size. *Eumpos perotis* and *Phyllostomus hastatus* scored high on this axis, indicating large and wide noses. The second axis (PC2, 15.5%) had a strong, positive loading on INWR (+0.906), and separated species with nostrils wide relative to head width (e.g., *N. macrotis* and *Tadarida brasiliensis*) from species with narrow set nostrils (e.g., *L. yerbabuenae* and *A. geoffroyi*). A third axis (PC3, 10.9%) was most strongly affected by NL (+0.587), separating species based on nose length. There was no particular pattern across clades or diet along the first axis (Figure 2.5a), although the insectivorous species tended to be smaller (positive loading), with fewer large bat species. Bats in the family Phyllostomidae tended to have high, positive loadings on

PC2, indicating narrow nostrils relative to head size. Across families, insectivores tended to have negative loadings on this second axis, suggesting relatively wider nostrils.

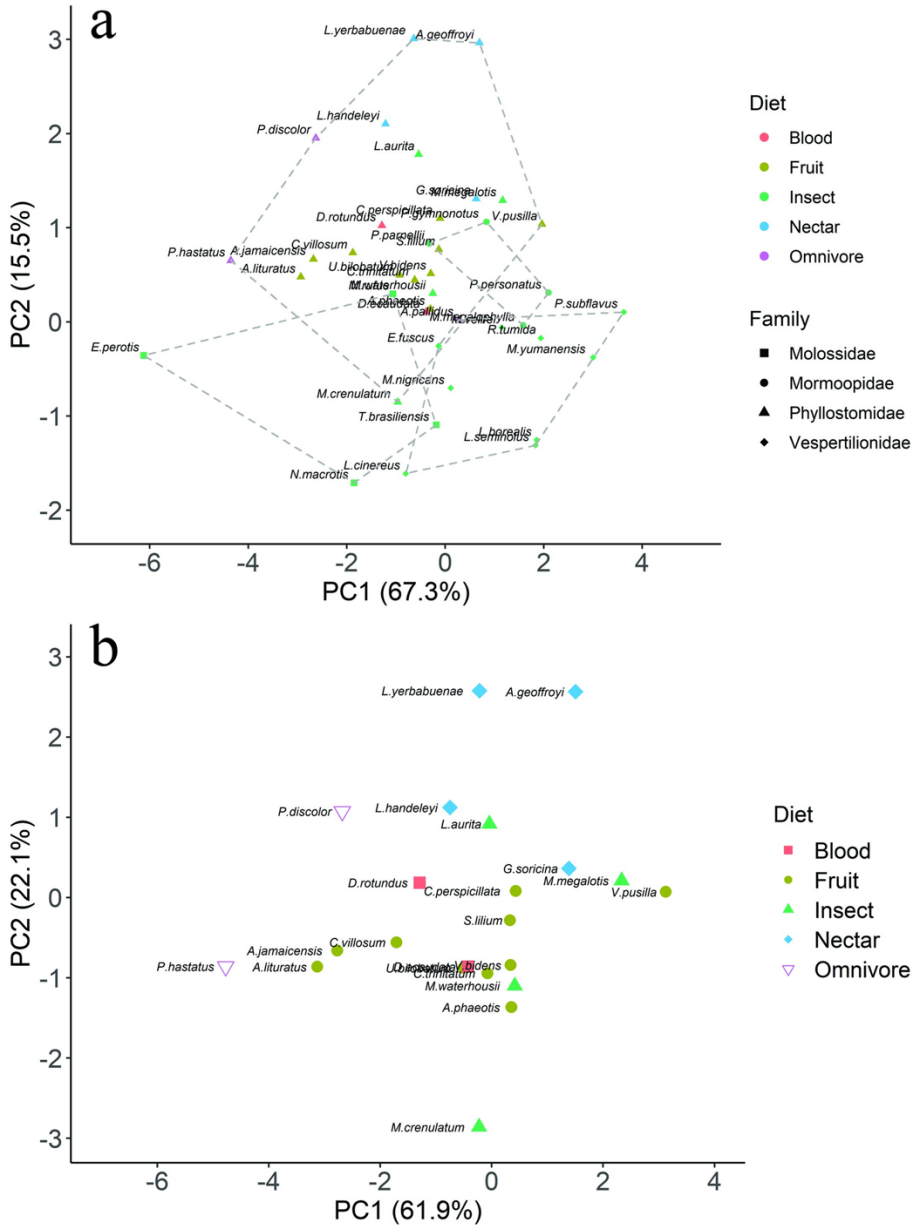

Figure 2.5. Ordinations of the first and second components of the phylogenetic principal component analysis (pPCA). pPCA results for the full dataset (a) and just Phyllostomidae (b). Dotted lines for the full dataset (a) delineate minimum convex hulls for each family.

A pPCA using only species in the family Phyllostomidae yielded similar patterns, with three component axes jointly explaining 91.9% of the variance. The first PC (61.9% of variance) was strongly affected by ONW (-0.895), NW (-0.828), CL (-0.922) and NareW (-0.939), with larger species scoring high on this axis (e.g., *Phyllostomus hastatus*). The second axis (PC2, 22.1%) also had a high, positive loading on INWR (+0.947), separating species with narrow set nostrils from species with wider nostrils. Most species fell intermediate to extremes on both axes, with no obvious pattern across different diets (Figure 2.5b).

2.3.3. Relationship between Morphology and Ecology

Using scores computed on the pPCA axes for each species, we constructed models to investigate the relationship between morphology and ecology. We ran multiple regressions with each principal component as a dependent variable, using either body mass or forearm as a covariate. For simplicity, we only present the results for regressions using body mass as the covariate, although regressions run using forearm as a size covariate produced comparable results (Appendix A). For PC1, models with the strongest statistical support included the independent influences of foraging habitat, echolocation mode and migratory behavior. The least complex models with higher statistical support (ΔAICc < 2) included foraging habitat, echolocation mode and migration pattern. Size was a significant predictor for all models ($P < 0.01$). Foraging habitat also had a significant effect on the response variable ($P < 0.05$), where species that forage in more open habitats tended to have larger head and body sizes (Appendix A). For PC2, the most relevant predictors were diet, foraging habitat and echolocation

mode. Diet was a significant predictor across all of the models with the most statistical support ($P < 0.001$). Nectar feeding bats had significantly higher loadings on PC2 compared to insectivores ($t = 5.73$, $P = 0.02$) (Figure 2.6a).

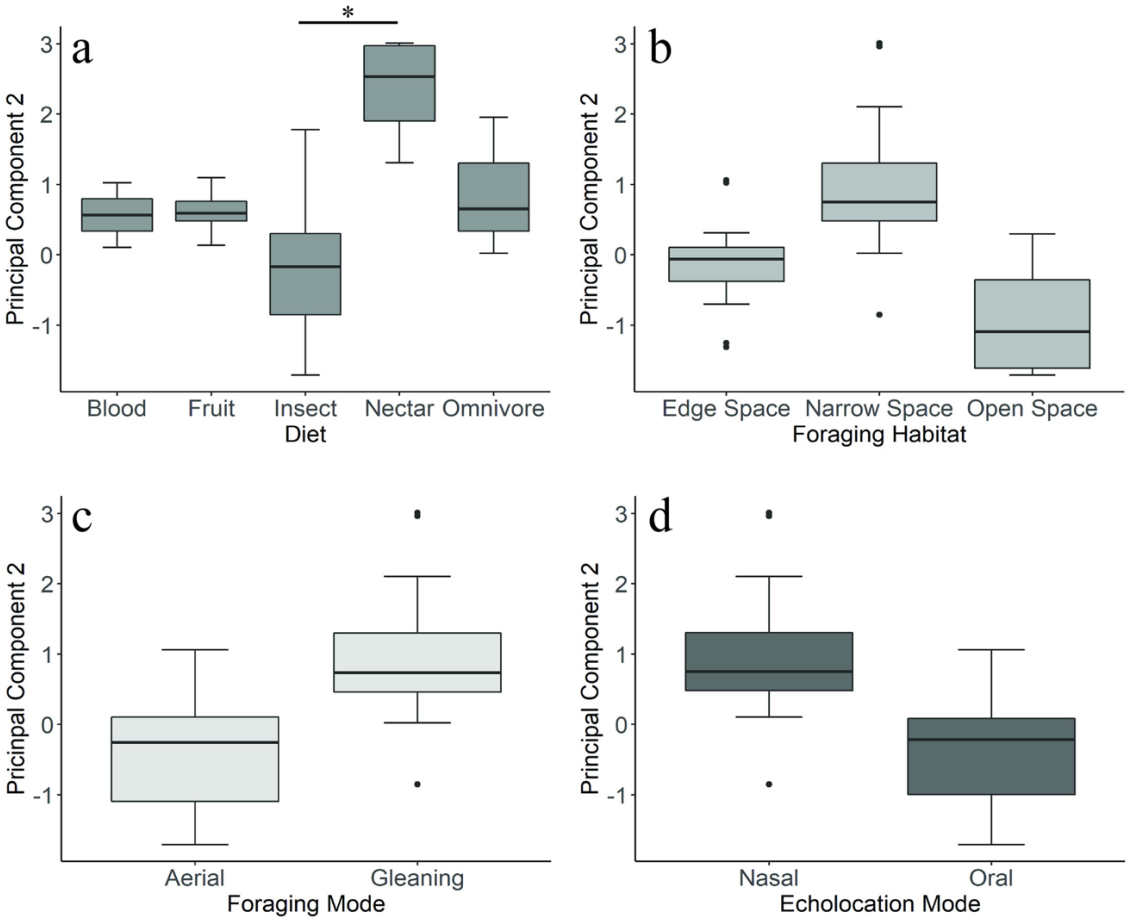

Figure 2.6. Boxplots representing variation in PC2. Data from the full dataset for four of the measured ecological variables: diet (*a*), foraging habitat (*b*), foraging mode (*c*), and echolocation mode (*d*). Only diet had a significant effect on PC2 in a PGLS regression. Asterisks indicate statistical significance in a phylogenetic ANCOVA.

Mean relative nostril width (INWR) differed significantly across bats with different diets (phylogenetic ANOVA, $F = 10.459$, $P = 0.018$). Bats that feed primarily on nectar had narrower nostrils compared to insect-eating bats (Holm-Bonferroni

adjusted, t = -5.19, P = 0.01, Figure 2.7a). Bats that forage via gleaning also had narrower nostrils compared to aerial foragers (F = 49.49, P = 0.018, Figure 2.7c), as did bats that echolocate nasally (F = 52.605, P = 0.017, Figure 2.7d).

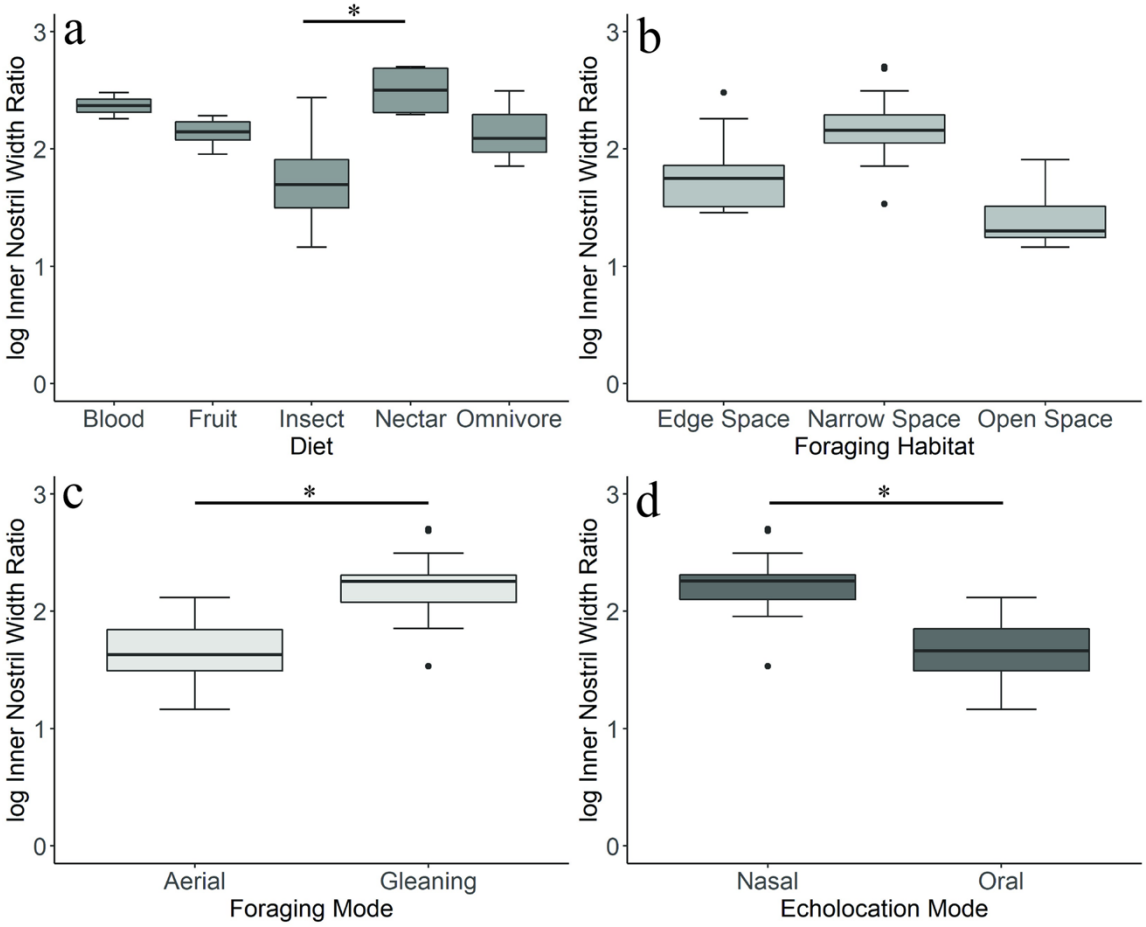

Figure 2.7. Boxplots representing variation in inner nostril width ratio (INWR). Data from the full dataset for four of the measured ecological variables: diet (a), foraging habitat (b), foraging mode (c), and echolocation mode (d). Asterisks indicate statistical significance in a phylogenetic ANOVA.

When comparing only between species in the family Phyllostomidae, diet was still a significant predictor for PC2 axis (P = 0.026), but inner nostril width ratios were not significantly different across diets (F = 4.39, P = 0.123) (Appendix A). Using PCs

and PGLS, we also tested for a relationship between morphology and quantitative flight characteristics (average speed, wing loading, and aspect ratio). There was no significant covariation between the scores of either principal component axis and any of the flight traits ($P > 0.05$) (Appendix A).

2.4. Discussion

Greater separation of the airstreams passing over the olfactory receptors is hypothesized to facilitate simultaneous comparison of the olfactory environment on opposite sides of the face, useful for olfactory tracking via tropotaxis. This may be especially true in organisms with limited post-cranial mobility or those moving at fast speeds in three-dimensional environments (Gardiner and Atema, 2010; Kajiura et al., 2005; Stoddart, 1979). Using bats as a model, we evaluated if there is a link between external nasal morphology and potential olfactory tracking behavior. Contrary to our predictions, bat species that rely on olfaction for foraging had narrower nostrils compared to species that rely primarily on echolocation or hearing for foraging, even when controlling for evolutionary history. It remains unclear what, if any, ecological factors may be driving the diversity of bat nasal morphology.

Phylogenetic history had a significant influence on the external morphological characters measured in this study, indicating that closely related species are more likely to resemble each other than distantly related species. However, this signal was reduced or non-existent when comparing external nasal morphology within the family Phyllostomidae, consistent with previous studies investigating the role of ecological

factors in driving morphological diversity in the family (Dumont et al., 2012; Freeman, 2000; Nogueira et al., 2009).

Contrary to our predictions, insectivorous bats across all families have wider nostrils (lower INWR), while nectar feeding species had the narrowest nostrils. This pattern was also detected even within just the family Phyllostomidae, as insect-eating species in our dataset trended towards wider nostril separation (although with a lot of variation). This is surprising given the well-documented use of olfaction by fruit and nectar-eating bats during foraging. In many species of plant-visiting phyllostomid bats, appropriate odor cues are necessary to stimulate foraging, even in the absence of other food-related cues (such as shape or texture) (Korine and Kalko, 2005; Thies et al., 1998; von Helversen et al., 2000). Bats are attracted to odor lures in the field, with more captures recorded in odor-baited mist nets (Mikich et al., 2003; Rieger and Jakob, 1988) and increased frugivore activity around fruit odor lures in open field areas (Bianconi et al., 2007). Olfactory cues may play a role in the detection of ripe fruits or flowers over long distances (Korine and Kalko, 2005; Parolin et al., 2019), as well as facilitating foraging in cluttered habitats (Muchhala and Serrano, 2015). However, it may be that bats rely more on spatial memory to locate potential food resources (Carter et al., 2010) and then rely on olfactory cues for fine-scale localization and discrimination.

Tropotaxis is not the only behavioral strategy animals might use to follow olfactory stimuli, and it may be that fruit and nectar feeding bats rely on other strategies to follow odor plumes while foraging. Increased maneuverability could compensate for narrow nostrils by allowing bats to quickly sample an area via klinotactic (serial

sampling) mechanisms. Frugivorous and nectarivorous species are generally well adapted for foraging in clutter habitats, with short, broad wings that allow for hovering and maneuvering around obstacles (Marinello and Bernard, 2014; Norberg and Rayner, 1987). Habitat also has an effect on the structure of odor plumes. Wind speeds tend to be lower in forested areas, creating longer bursts of odor signals in the air over father distances (Murlis et al., 2000). However, vegetation can also cause continual shifts in wind and odor direction, making plume following more difficult (Elkinton and Cardé, 1984). Under these conditions, behavioral rather than morphological adaptations would be needed to support odor tracking.

Morphological adaptations for odor tracking may be constrained by selection for other purposes. Multiple studies in phyllostomid bats have shown that adaptive shifts in cranial size and shape as well as bite force are strongly associated with feeding mechanics (Dumont et al., 2014; Nogueira et al., 2009; Santana et al., 2010). In frugivorous species, this is frequently characterized by shorter rostra and mandibles and more robust crania (Freeman, 2000; Nogueira et al., 2009), while nectarivorous bats instead display elongated rostrums, thought to be associated with the development of an elongated tongue (Dumont et al., 2014; Nogueira et al., 2009; Santana et al., 2010). While cranial and external soft tissue morphology are likely correlated, the extent to which these units might evolve together is unknown. Thus, it is possible that while natural selection acted on cranial morphology associated with the manipulation and processing of food, there was less selective pressure on external characteristics, leading

to a mismatch in features resulting in larger heads or longer noses while keeping external characteristics such as nose shape the same.

Feeding ecology is unlikely to be the only driver of this morphological variation in external nose morphology. Within our dataset, diet only explained about 36% of the variation in external nasal morphology, and foraging habitat explained only 11% of total variation. All of the fruit and nectar feeding species in this dataset are found within the family Phyllostomidae, a group of nasal-emitting echolocators. Nasal emitting bats were shown to have significantly narrower nostrils relative to head size than oral emitting bats. This presents an interesting potential trade-off between sound emission and olfactory tracking via tropotaxis in nasal emitting bats. While nostril separation in long duration constant-frequency emitting bats (Rhinolophidae and Hipposideridae) is tightly linked to echolocation parameters (Pye, 1988), it is unknown if the short broadband, frequency modulated calls of phyllostomid bats are similarly influenced by nose morphology. Future work comparing these distinct groups could help disentangle the selective pressures of nasal echolocation on external nose morphology. Bats in the family Pteropodidae are also known to rely on olfactory cues while foraging (Hodgkison et al., 2007; Luft et al., 2003; Raghuram et al., 2009; Sánchez et al., 2006), but do not echolocate laryngeally. It might be interesting to further test the relationship between foraging ecology and external nasal morphology within those species, without the confounding effects of echolocation.

External nare shape and orientation likely play an important role in the control of airflow into the nasal cavity, for both respiration and olfaction (Clifford and Witmer,

2004; Craven et al., 2007; Ranslow et al., 2014). During sniffing in dogs, air is inhaled from the front and exhaled to the side, which alters airflow rates and permits more efficient sampling of odorants (Jenkins et al., 2018). Interestingly, bats appear to be different from rodents and dogs in that some air may pass through the olfactory recess during both inhalation and exhalation, thus potentially increasing odorant absorption on olfactory epithelium (Eiting et al., 2014). In addition to differences in width and size, bats also display considerable variation in shape and orientation of external nares (Fig 2.1). While linear measurements such as those presented in this study can be reliable indicators of size variation, they are unlikely to completely reflect shape variation in shape (Fabre et al., 2014). Although geometric morphometric approaches are better at capturing shape data in morphological studies, the lack of consistent landmark features in soft tissues makes this technique difficult. Using advancements in three-dimensional imaging and reconstruction such as diceCT (Yohe et al., 2018) or spiceCT (Witmer et al., 2018), future work could investigate how this morphological variation might influence nasal airflow and thereby olfactory behavior.

Molossids (free-tailed bats) have the widest nostrils compared to other insectivores, even when accounting for differences in body size. Larger and wider nostrils may be advantageous for these high, fast flying species with high respiratory demand (Negus, 1954). Molossids are also known for their strong odors; males of many free-tailed bats species (including all four molossid species used in this study) develop gular-thoracic glands that may be used to mark females or roosting sites (Keeley and Keeley, 2004; Scully et al., 2000). It is possible that even if these species are unlikely to

be using olfaction as a sensory cue while foraging, they may use olfactory cues to find potential mates, or as homing cues during migration, as observed in some species of seabirds (Abolaffio et al., 2018; Reynolds et al., 2015; Safi et al., 2016).

Nose and nostril morphology are also influenced by respiratory and thermoregulatory demands (Negus, 1954), although how these demands interact with sniffing and olfaction is still unclear. In bats, the respiratory cycle is closely related to the wing-beat cycle, and echolocation pulses are generally emitted during expiration (Falk et al., 2015; Roberts, 1972; Suthers et al., 1972). Sniffing, or bouts of increased air intake, is often associated with exposure to olfactory stimuli. Mammals, including rodents, moles, and bats will increase their sniff rates in response to olfactory stimuli (Laska, 1990a; Wesson et al., 2008a). Sniffing has only been rigorously testing in stationary bats, so it is still unknown how they balance olfactory inputs with respiratory demands, or how much they are able to sniff while flying.

Several experimental studies have demonstrated the importance of stereo-olfaction for scent-tracking in rodents (Khan et al., 2012; Liu et al., 2020; Rajan et al., 2006), which are comparable in size to some bat species. Across all bat species in this study, the average inner nostril width ratio was 7.6, compared to 6.0 (from (Stoddart, 1979)) and 7.0 (this study, Table 2.2) for rodents. Among the bat species in this study, insectivorous molossid and vespertilionid bats had wider relative nostril widths even compared to rodents (Table 2.2). Studies on the computational fluid dynamics of airflow during sniffing in dogs and humans suggests that extremely wide nostrils are not necessary to take advantage of separate sampling areas (Craven et al., 2010; Porter et al.,

2007; Staymates et al., 2016). During inspiration, air in the vicinity of the nostril is drawn towards the naris, creating a hemispherical region in front of the naris called the 'reach' of the nostril. In canines, the reach of a nostril is approximately 1 cm, which is smaller than the inter-nostril separation, indicating that each nostril is sampling air from spatial separate regions (Craven et al., 2010). In humans, each nostril can sample information from areas that are separated by about 3.5 cm (Porter et al., 2007), which is wide enough to span the boundary of a scent plume (which can be within 10 mm; Crimaldi and Koseff, 2001). Similar computational studies have not been done in rodents or bats, so it is unknown how these values might scale down to smaller animals. Narrow nostril widths in nectar feeding species does not preclude these species from using bilateral sampling mechanisms for olfactory localization, but wider widths in insectivorous bats suggest they may use olfactory tropotaxis more than expected.

Table 2.2. Average inner nostril width ratios (INWR) and standard error (SE) for a sample of mammalian taxa.

Taxa	INWR	± SE	Source
Insectivores Marsupials Tree shrews	5.1	0.69	Stoddart 1979
Rodents	6.0	0.41	Stoddart 1979
Rodents	7.0	0.40	this study
Rattus rattus	6.6	1.22	this study
Mus musculus	7.4	0.71	this study
Bats	7.6	0.45	this study
Molossidae	4.5	0.78	this study
Mormoopidae	7.0	0.44	this study
Phyllostomidae	9.5	0.48	this study
Vespertilionidae	4.9	0.23	this study

The relationship between morphology and olfactory ecology in bats is complicated by the tangential interactions between breathing, feeding, and echolocating, which can lead to compromises in the various physiological and mechanical parameters of the nose and rostrum. To our knowledge, this is the first study to evaluate the role of ecology in shaping the morphology of an external sensory character, using modern phylogenetic comparative methods. We found that nectar eating bat species have narrower nostrils than insect-eating species, and that nasal echolocation may impose constraints on tropotactic mechanisms for olfactory tracking in bats. Alternatively, the results also indicate that some insectivorous bats, like the Molossidae, may rely upon stereo-olfaction more than expected. Pairing morphology and physiological studies of olfaction with behavioral studies quantifying the patterns of olfactory tracking in bats will provide more insight into how bats integrate olfactory information while foraging.

3. OLFACTORY TRACKING STRATEGIES IN A NEOTROPICAL BAT

3.1. Introduction

Olfactory search trajectories show striking similarities across diverse taxa suggesting that many species have converged upon a similar sequence of behaviors to solve the problem of locating an odor source in a dynamic environment (Ache and Young, 2005; Svensson et al., 2014). Examples from many animals have revealed a multi-tiered search strategy to detect and follow odors to their source that relies upon a combination of serial sampling (klinotaxis) and zig-zag 'casting' behaviors far from the source that is replaced by more side-to-side head scanning movements and stereo-olfaction (tropotaxis) when near the odor source (Baker et al., 2018; Catania, 2013; Liu et al., 2020; Louis et al., 2008; Thesen et al., 1993). Bats offer an interesting test of the generality of this behavioral sequence in mammals because of their aerial nature, high speeds, and potential morphological and physiological constraints associated with echolocation.

Olfactory cues play a key role in foraging by frugivorous and nectarivorous bats (Korine and Kalko, 2005; Rieger and Jakob, 1988; Thies et al., 1998; von Helversen et al., 2000), but the extent to which bats rely upon olfaction to find food is still unknown. Olfaction was shown to be an important cue for detecting the presence of ripe fruit in *Carollia* (Leiser-Miller et al., 2020; Thies et al., 1998). Neotropical fruit bats are highly sensitive to fruit odors and can discriminate odor qualities and quantities; the first step in being able to recognize a concentration gradient (Laska, 1990a; Laska, 1990b). Many

bats use olfaction in combination with echolocation but appear to rely mainly on sonar cues to locate targets once stimulated by the presence of an attractive odor cue (Korine and Kalko, 2005; Thies et al., 1998). Consequently, the abilities and limitations of bats to track an odor source exclusively by olfaction remains to be determined.

The chemical gradient emanating from a single source is predicted to follow a Gaussian distribution, with the precise odor structure dependent upon molecular weights, diffusion coefficients and emission rates of the odor cocktail, as well as wind speed, atmospheric stability, and distances from odor source (Elkinton and Cardé, 1984). Time-averaged models of odor concentration predict a steep gradient near the odor source that transitions to a shallower gradient farther away from the source (Elkinton and Cardé, 1984; Elkinton et al., 1984; Louis et al., 2008). At distances farther from the odor source, where the odor gradient is shallow or irregular (with peaks in instantaneous concentration; Murlis et al., 2000), animals use klinotaxis to orient towards a chemical source by sequentially sampling as they move through the environment (Dusenbery, 1992). Closer to the source where the odor gradient becomes steeper, animals can also exploit the simultaneous comparisons of odor intensity (Catania, 2013; Takasaki et al., 2012) or arrival timing (Gardiner and Atema, 2010) between two or more spatially segregated receptors (tropotaxis). Morphological comparisons of nostril widths in bats suggest that the nasal emission echolocation pulses may impose an important constraint on leaf-nosed bats' abilities to exploit tropotactic mechanisms (Brokaw and Smotherman, 2020), leading us to hypothesize that they may rely more heavily on other or different behavioral strategies to track odors.

Animals can optimize their olfactory search behaviorally, particularly in response to environmental variations in odor concentrations or plume turbulence. Animals commonly reduce their speeds when navigating in more turbulent flows, a pattern observed in a range of taxa including crabs (Weissburg and Zimmer-Faust, 1994), lobsters (Moore et al., 1991), dogs (Thesen et al., 1993), and coati (Hirsch, 2010). Animals can adjust their sampling strategies for olfactory cues by increasing rates of sniffing (Khan et al., 2012; Porter et al., 2007), antennule flicks (Koehl, 2006), or by lateral movements of the head, nose, or antenna (Gomez-Marin et al., 2010; Ino and Yoshida, 2009; Khan et al., 2012; Liu et al., 2020; Mathewson and Hodgson, 1972; Porter et al., 2007; Thesen et al., 1993). Animals can also incorporate a combination of sensory and cognitive strategies (i.e., learning and spatial cues). We refer to this complex behavior as "*route-following*" to reflect that the animals can learn the spatial arrangements of their environment (natural or experimental) and can deduce the most efficient routes for inspecting multiple likely source coordinates. For example, mice can use airborne gradients to locate odor rewards, but find rewards faster when relying instead on previous experience (Gire et al., 2016). Bats are known to use spatial memory while foraging (Fleming et al., 1977; Thiele and Winter, 2005) and so may also be able to combine olfactory cues with spatial information to locate odor sources.

In this study, we quantitatively analyzed the locomotor patterns and behavioral strategies of a phyllostomid bat (Chiroptera: Phyllostomidae) searching for an attractive odor source while crawling downwards. We chose to focus on the northern yellow-shouldered bat, *Sturnira parvidens* (Goldman, 1917) (Figure 3.1A) because of its diet,

wide distribution, and its use of olfaction for social communication (Faulkes et al., 2019; González-Quiñonez et al., 2014). The northern yellow-shouldered bat is a small frugivore (13 – 18 g) common to much of Central America (Hernández-Canchola et al., 2020). This species feeds on a variety of fruits, including banana, wild fig (*Ficus*), and neotropical fruits in the genus *Solanum* (including *S. hazenii, S. angulate, S. americanum* and *S. torvum*; Castro-Luna and Galindo-González, 2012; Fleming et al., 1977). Field observations suggest these bats may first use olfactory cues in flight to identify trees bearing ripe fruit, prompting them to land and crawl along branches where they may rely upon olfaction to find fruit obscured by foliage. Preliminary behavioral experiments confirmed that *Sturnira* readily sought out food in an experimental setting without requiring extensive training, and thus could provide a useful model for measuring bat olfactory tracking capabilities and characterizing their locomotor search strategies. First, we established that naïve crawling bats would successfully localize an attractive odor source in the absence of salient biosonar cues. We then analyzed the locomotor search patterns by quantifying trajectories, speeds, and head-scanning behaviors throughout the search to provide a comprehensive characterization of their odor localization strategies across experimental conditions.

3.2. Materials and Methods

3.2.1. Field Conditions

We conducted field experiments from April 23 – May 2, 2019, at Lamanai Outpost Lodge, Orange Walk, Belize (17°45′N; 88°39′W). Bats were captured using mist-nets from along forest trails and clearings in the Lamanai Archeological Reserve

(within 2 km of the Lodge). On the night of capture, we placed individual bats in the experimental arena for between 1 – 2 hours with several pieces of banana in plastic hexagonal weigh boats on the floor of the arena. Only individuals that spontaneously sought out and consumed the banana reward by the end of this trial period were retained for behavioral experiments, resulting in N = 10 male bats. We only used adult male bats in this study to reduce potential confounding factors of sex or age.

3.2.2. Ethical Note

Experiments were carried out under permits from the Belize Forestry Department (permit number FD/WL/1/19 (10)) and were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP # 2017-0139). Between experiments, bats were housed together in soft mesh cages (60.9 x 60.9 x 91.4 cm) in a dark, quiet location and provided water ad libitum. During the first 24 hours following capture, bats had access to small bowls containing ripe banana at the bottom of the cage. We released all bats at their capture site after a maximum of five days.

3.2.3. Experimental Assays

We measured olfactory localization behavior in naïve bats using a two-choice olfactory assay and standard operant procedures. The testing arena was a soft mesh cage (37 x 37 x 71 cm) oriented vertically to allow bats to hang and move naturally (Figure 3.1B). Pilot behavioral experiments conducted in Belize in 2018 found that bats were more motivated to investigate a possible food reward when allowed to crawl vertically as opposed to crawling horizontally on a surface, as this more closely mimics natural hanging and crawling conditions (such as might be seen in a roost). The front face of the

cage was made of clear plastic to allow video recording. Experiments took place between 20:00 and 06:00 hours and were video-recorded with a Basler Ace model ac640-um digital video camera connected to a laptop running Basler Video Recording Software (Ahrensburg, Schleswig-Holstein, Germany). Videos were recorded at 30 frames per second and 640 x 480-pixel resolution. We ran all experiments in complete darkness, except for illumination with infrared LED light strips attached to the sides of the arena, to remove any confounding visual cues. At the beginning of each trial, bats were placed at the top center of the arena, and stimuli were presented in small plastic bowls (2.5 cm diameter weigh boats), placed at the bottom on opposite sides of the arena. Positively reinforced stimuli (S+) included real banana pieces or a chemical olfactory cue mixed with sugar water. Chemical olfactory stimuli were prepared using food-grade banana baking emulsion, composed of artificial and natural flavors (LorAnn Professional Kitchen, Michigan, USA). We prepared four concentrations of banana solution using serial dilution, adding 1 ml of banana emulsion (or resulting dilution) to 9 ml of 30% (w/w) sugar solution. All dilutions were prepared from the same batch of banana emulsion and sugar solution. Neotropical bats can discriminate between natural and artificial banana odor (Laska, 1990b), but will still readily consume artificial banana (A. Brokaw, personal observations). We chose to use a baking emulsion as an olfactory cue instead of a pure chemical compound (such as isoamyl acetate) to allow bats to safely consume or taste a reward, in order to maintain motivation during the behavioral trials. During the acclimation and initial training period following capture, we presented bats with banana pieces supplemented with 10% banana-sugar solution to ensure that

bats associated the artificial olfactory stimulus with the real banana reward. Unreinforced stimuli (S-) were distilled water or an unflavored piece of sponge cut to mimic the shape and texture of a piece of banana.

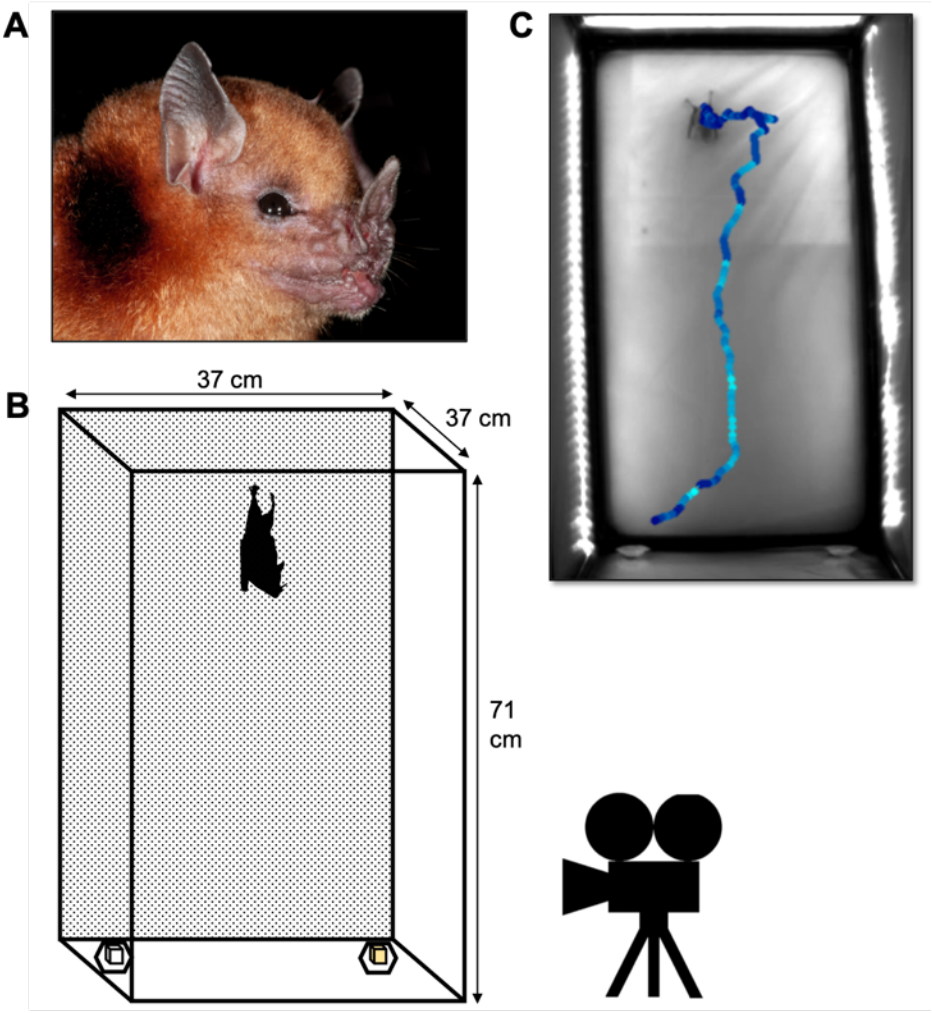

Figure 3.1. A) Photograph of the study species, northern yellow-shouldered bat (*Sturnira parvidens*). Image by Brock and Sherri Fenton, used with permission. B) Diagram of experimental arena used to test bat localization behaviors. The back part of the arena is made of soft mesh for bats to comfortably hang and crawl downward, while the front panel is clear plastic to allow video recording. Shapes at the bottom represent the olfactory stimuli: yellow (S+) and white (S-). C) Example video still and resulting movement track, extracted from EthoVision XT 13. Color gradient represents the bats' instantaneous velocity, with lighter tones representing faster movement. Image has been cropped for visualization purposes.

In preliminary experiments, we placed a condenser microphone (model CM16, Avisoft Bioacoustics, Berlin, Germany) at the base of the arena to record their echolocation behavior during the olfactory searches. We only detected their broadband echolocation calls when the bat was very near to and directly facing the microphone. However, we noted that the nose-leaf twitched every time the bat emitted a pulse and based on this, it was evident that the bats were continuously emitting pulses whenever they were moving. Since we could not reliably record the pulses throughout the arena as the bats moved we did not try to quantify their echolocation beyond confirming that they actively echolocated throughout all trials.

The following experiments were designed to evaluate the olfactory search behaviors used by bats locating an odor cue. The first experiment was designed to ensure that naïve bats would reliably seek out a familiar food reward possessing a strong olfactory cue in the test chamber. In the second set of experiments, we controlled for the possible effects of echolocation during olfactory search by testing if bats could locate the S+ in the absence of salient sonar acoustic cues, by presenting an unscented shape or removing shape cues completely. In the third experiment, we tested the effect of changing odorant concentration on the bat's olfactory localization performance (Table 3.1). Lastly, we used bat movement trajectories from all experiments to quantitatively describe the behavioral search strategies of crawling bats.

Table 3.1. Summary of behavioral experiments to test odor localization behaviors in crawling bats.

	Treatment	Rewarded stimulus (S+)	Unrewarded stimulus (S-)
Experiment 1:			
Can bats localize a food-reward by its odor?	Banana vs Shape	Banana cube + 10% odor solution	Sponge cube + distilled water
Experiment 2:			
Is odor source localization dependent upon sonar cues?	Shape vs Odor	10% odor solution	Sponge cube + distilled water
	Odor vs No Odor	10% odor solution	Distilled water
Experiment 3			
What effect does odor strength have on bat localization performance?	0.1%	Sponge cube + 0.1% odor solution	Sponge cube + distilled water
	1%	Sponge cube + 1% odor solution	Sponge cube + distilled water
	10%	Sponge cube + 10% odor solution	Sponge cube + distilled water
	100%	Sponge cube + 100% odor emulsion	Sponge cube + distilled water

3.2.3.1. Acclimation and Training

On the first night after capture, we introduced naïve bats to the arena and gave them up to two hours to explore the cage and find the banana food rewards. Bats were gently repositioned by hand at the top of the arena each time a new piece of banana was added to the dish to acclimate them to being handled and reinforce the goal-seeking behavior. Most, but not all, bats quickly learned the task after one night, allowing experimental trials to begin on the second night. Bats that did not seek out food within the arena on the first night were released at their capture site the following night. To reinforce the behavior each night, each experimental session began by presenting the

bats with two banana pieces supplemented with 0.5 ml of 10% banana extract solution, which was done to ensure that the bats would associate the extract banana smell with real banana reward even if the bats perceived a difference between extract and real banana smell. We allowed bats to explore the arena until they located and consumed both pieces of banana. We recorded the location where the bat found the first banana. For the following experimental trial, the olfactory stimulus was switched to the opposite side to discourage side bias.

3.2.3.2. Experimental Animals

On a given night, in-between trials, bats were held individually in soft, cloth bags in a quiet area. Each night, we randomized the order that individuals were tested. The arena was wiped with 95% ethanol and allowed to dry between trials to reduce confounding odor cues. Although experiments are presented and analyzed separately, the trials for all three experiments were randomized within and across nights, to avoid potential confounding effects of learning and maximize sampling across limited individuals and time. We aimed to test each individual ten times at each treatment. Trials with a banana reward were arranged to be every 4th or 5th trial, to ensure bats sustained motivation, and so were repeated more than 10 times per individual. The location of S+ was pseudo-randomized for each trial, with its position repeated no more than three consecutive times. We carried out trials under ambient airflow conditions, and temperature and relative humidity were recorded at the start and end of each trial.

3.2.3.3. Experiment 1: Localization of food reward using odor

During this experiment, bats had the option to choose between a banana reward (S+) and control object (S-). Both choices were placed in plastic weigh boats at the bottom of the arena. We cut ripe bananas into cubes, approximately 1 cm^3. The control object was a cosmetic sponge cut into the same 1 cm^3 shape as the banana piece. We supplemented the banana reward with 0.1 ml of 10% banana-sugar solution. Both stimuli were prepared and placed in the arena immediately prior to the start of the behavioral trial. We placed an individual bat at the top of the arena to start the trial. Trials lasted until the bat located and consumed the piece of banana, or after a maximum of five minutes had elapsed. If bats did not attempt to feed on the banana after five minutes, then a "no-choice" result was logged.

3.2.3.4. Experiment 2: Role of acoustic cues during reward localization

The following treatments were designed to isolate olfactory cues from acoustic cues and determine whether or not both sensory modalities (acoustic or odor) were necessary or preferred by the bats during odor localization. In Experiment 2A, we placed 0.5 ml of 10% odor-sugar solution alone (S+) in a plastic weigh boat on one side of the arena, while the other side of the arena held an unscented cosmetic sponge cube cut to resemble a piece of banana placed in 0.5 ml of distilled water (S-). This was designed to test which cue type (odor or acoustic) was more important in the bat's search behaviors. In Experiment 2B, we tested how well bats could localize an odor when there was no salient acoustic cue (cosmetic sponge) by placing 0.5 ml of 10% odor-sugar solution (S+) on one side of the arena, while the other side held 0.5 ml of distilled water (S-). If

bats were successfully able to locate the odor cue, this would provide strong evidence for localization using only odor cues. Trials began when we placed a bat at the top of the arena, and continued until the bat touched, grabbed, or licked one of the stimuli. If bats did not select either target after five minutes, then a "no-choice" result was logged.

3.2.3.5. Experiment 3: Effect of odor strength on localization success

To evaluate whether or not odor concentration influenced localization performance or search strategies, we challenged the bats with four different concentrations of banana odors. During these experiments, we placed two cosmetic sponge cubes (1 cm^3) in plastic dishes on opposite sides of the arena. One of the sponges held 0.1 ml of odor-sugar solution (S+), while the other side held a sponge and 0.1 ml of distilled water (S-). We tested bats with four different odor concentrations: 100% (only banana extract), 10%, 1% and 0.1%. We determined bats made a choice when their nose or mouth touched the sponge or weigh boat of one of the stimuli. If bats did not select either target after five minutes, then a "no-choice" result was logged.

3.2.4. Behavioral Scoring and Movement Analysis

We recorded every trial for all experiments to analyze and reconstruct the locomotor patterns and pathways used by the searching bats. This information can reveal whether or not the bats consistently used any of the previously defined search strategies seen in other animals (i.e., cast and surge) while tracking odor sources across experimental contexts. We extracted and analyzed bat locomotor patterns and two-dimensional trajectories using Noldus EthoVision XT 13 (Leesberg, Virginia, United States, Figure 3.1C). The coordinate space was calibrated automatically in EthoVision

XT by inputting the real-world height and width of the back of the experimental arena (where bat movement would be measured). The coordinate space was calibrated individually for each video, to account for any movements of either the arena or camera between trials. Bat choices was determined when a bat touched their nose or mouth to one of the stimuli (touching either banana, sponge, or weigh boat) Trials were scored a 'success' when bats correctly chose the side with the S+. For each trial, we measured or calculated the following: start distance (cm), total distance travelled (cm), average velocity (cm/s), path straightness, decision distance (cm), and path shape (Table 3.2). Total distance travelled and average velocity were automatically calculated in EthoVision XT. We manually measured or classified starting distance, decision distance, and path shape from each trial using the integrated tracking view in EthoVision XT. Starting distance and decision distance was calculated in EthoVision XT as the straight-line distance between the bat center point and the odor location at start of the trial (starting distance) and at the time point where the bat made its last change of direction before moving towards its target (decision distance). To investigate if and how bats use head movements during an olfactory localization task, we also analyzed head scanning behavior for successful trials in Experiment 1 and Experiment 3. A head scanning event was counted each time the bat rotated its nose at least 45 degrees off axis to one side or the other and were only observed to occur consistently when the bat was stationary. Actively crawling bats generally kept their nose leaf pointed forward in line with the body axis; during locomotion any changes in head orientation were coordinated with concurrent changes in body orientation and therefore not interpreted as head scanning.

We extracted the distance from the odor source at which each head scanning event occurred using EthoVision XT. In addition to counting total number of head scanning events, we also recorded the number of head scans that occurred before or after the bat started moving towards the bottom of the arena (starting distance), and before and after the bat made its final decision (decision distance). Head scanning events at the start or decision distance were counted as occurring 'after' this cutoff.

Table 3.2. Description of behavioral parameters measured from trial recordings for all three experiments.

Pathway Analysis	Definition
Start Distance (cm)	The straight-line distance from the bats' starting position to the center of S+
Distance travelled (cm)	The total distance a bat crawled in the arena before making contact with either S+ or S-
Average velocity (cm/s)	The velocity of the bat crawling in the arena, averaged over the entire trial time.
Path Straightness	A value between 0 and 1 that indicates how directly the bat moved towards its choice, calculated as the ratio of the bats' starting distance from its choice (either S+ or S-) to the total distance travelled. Values close to 1 indicate a direct route while values close to 0 indicate a more meandering path.
Decision distance (cm)	The straight-line distance from S+ at which the bat made its last change of direction before moving towards its target (either S+ or S-)
Head Scanning Behavior	The number of times a bat performed a lateral movement of the head. A single head scanning event was counted each time the bat rotated its nose at least 45 degrees to one side or the other.
Path shape	Visual classification of the paths followed while navigating towards the target. Trajectories were classified qualitatively into four categories: *top casting, direct, middling, and bottom casting.*.
Top casting	Bat moves horizontally along the top of the arena before making direct downward movement to its final choice.
Direct	Bat executes a direct, downward movement starting from the location where it was located at the start of the trial.

Pathway Analysis	Definition
Middling	Bat initially moves downward but alters trajectory between one-third to one-half of the way down the arena.
Bottom casting	Bat makes direct movement downwards on one side of the arena, but pauses directly above target and redirects to alternate target, moving horizontally towards its final choice. Paths of this type have a distinctive "L" shape.

Only trials where bats remained along the back of the arena until making a choice were used for trajectory analysis and classified into path shapes (Figure 3.2A). Due to inaccuracies in tracking introduced by three-dimensional motion, trials where bats flew or hovered during the trial, or crawled along the side panels of the arena were excluded from trajectory analysis, although these trials were included in the analyses of bat success rates.

Path shapes were qualitatively classified visually from the detailed tracking view in EthoVision XT 13, and trajectories were defined as one of four categories: top casting, direct, middling, and bottom casting (Figure 3.2A, Table 3.2). Top casting was defined by horizontal movement from the bats' staring position at the top of the arena, in which bats crossed the midline of the arena at least once before making a straight path downwards towards one of the stimuli. In direct strategies, bats moved downward without making horizontal shifts in movement. These paths were either straight downward or had a slightly diagonal shape, depending on the bat's exact starting point. Bottom casting strategies were essentially the inverse of top casting paths, in which bats made a straight movement downwards towards one of the stimuli, but then moved horizontally across the bottom of the arena (crossing the midline at least once) before

making a final choice. Paths in this category produce a distinctive “L” shaped pattern. The middling strategy was characterized by general meandering of the path across the arena, in which bats shifted towards the middle of the arena while moving downwards, and then angled diagonally to one of the stimuli between one-third to one-half the way down the arena (vertical distance).

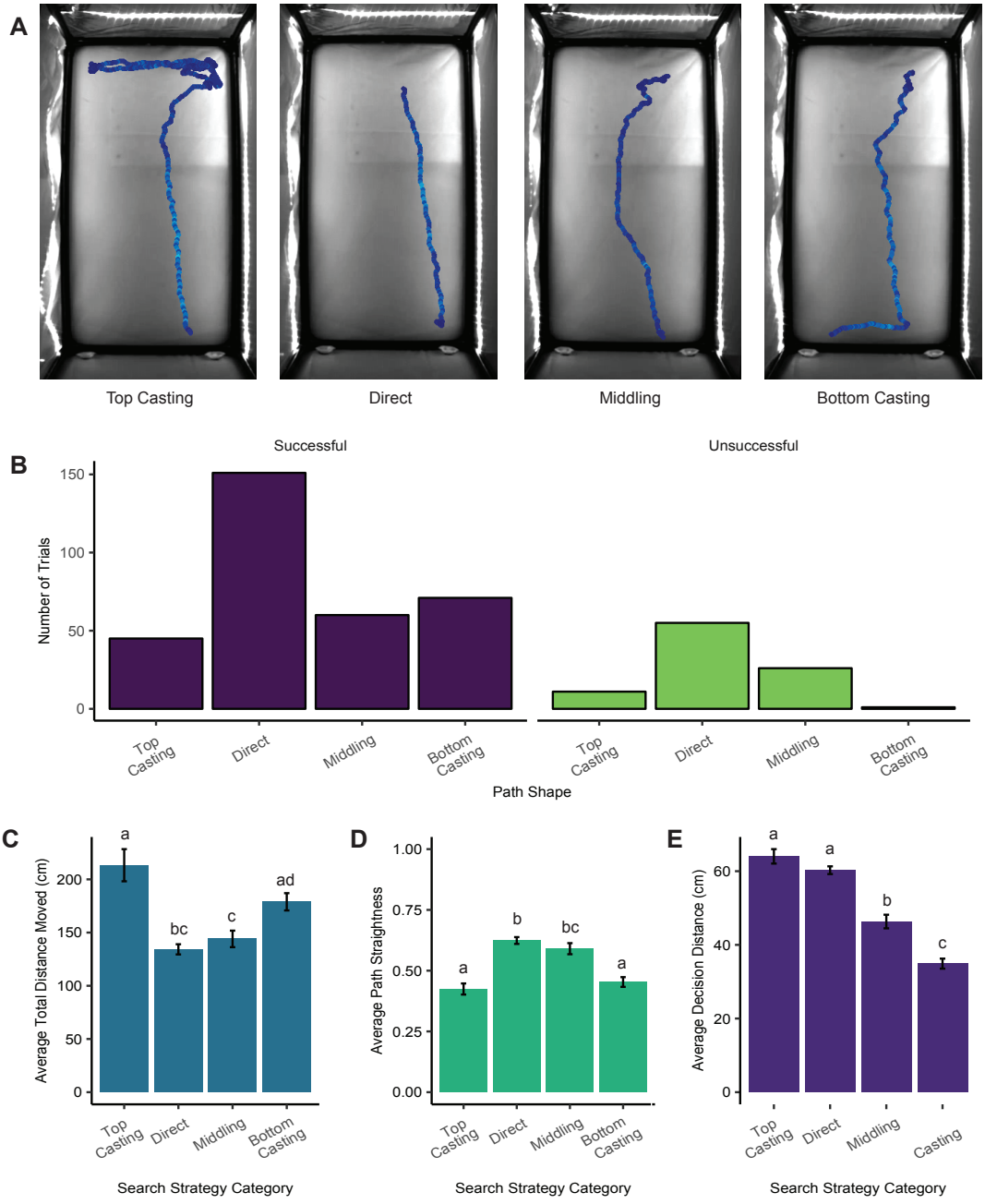

Figure 3.2. A) Example tracks for each of the four path shapes (Table 3.2). These tracks were selected from different individuals and different experimental trials. Images have been cropped for visualization purposes. B) Distribution of the observed number of trials for each path shape for successful (n = 327) and unsuccessful (n = 93) trials, pooled across all treatments and individuals. C) The average total distance traveled by bats for each search strategy category. D) The average path straightness (ratio between starting distance from correct stimuli and total distance moved) across the search strategy

categories. Values closer to 1 indicate a straight-line trajectory. E) The average distance at which bats made their final decision across all four search strategy categories. Error bars for C-E represent within-in individual standard error ('summarySEwithin' in package 'Rmisc', Hope, 2013). Means with the same letters are not significantly different according to Tukey's post-hoc tests (repeated measures ANOVA, $\alpha = 0.05$).

3.2.5. Estimating the Odor Concentration Gradient

To estimate the distribution of odors in the arena, we recreated the field setup in the lab (College Station, Texas, United States) to measure odor concentrations using a handheld photoionization detector (PID) (PhoCheck Tiger, Ion Science, Royston, United Kingdom). We placed the same type of plastic weigh boat used in field trials and containing 0.1 ml of 100% banana extract on one side of the olfactory arena, at the same location where the odor stimuli were placed during behavioral trials. We divided the back of olfactory arena into 120 grid spaces, each approximately 5.5 cm^2. Each grid space was measured at 1 second intervals for 5 seconds, and values were averaged for each space. The PID was set to use isoamyl acetate as a standard and was zeroed in clean air using a carbon filter attachment immediately prior to measurements. Since measuring the entire arena would take longer than the maximum time bats were in the arena, we also took measurements of the horizontal and vertical odor distributions at time point zero (immediately following placement of the odor in the arena) and after five minutes, representing the start and end conditions of each trial. While the lab environment is expected to be different from field conditions, the purpose was not to recreate the precise olfactory environment bats may have been exposed to, which undoubtedly varied slightly between trials, but rather to provide a general estimate for how odors may be distributed within the arena.

3.2.6. Statistical Analysis

The percentage of trials that the bat correctly chose the odor stimuli (S+) were taken as a measure of performance in all three experiments. Trials where bats did not select either stimuli (no-choice) were excluded from analysis. Bat performance between treatments was analyzed using generalized linear mixed models (GLMM) with a binomial distribution (using glmer in the 'lme4' package in R; Bates et al., 2015). Bat ID was included as a random effect to account for repeated testing of individuals. We first tested if environmental conditions (temperature and humidity) significantly influenced bat performance. We averaged the temperature and humidity for each trial ([start value + end value]/2) and analyzed their effect using a GLMM, with temperature and humidity as fixed effects. Post-hoc tests for significant variables ($P < 0.05$) were carried out using Tukey contrasts, adjusted for multiple comparisons (glht in package 'multcomp'; Hothorn et al., 2008). To test if the bats were overall able to discriminate better than chance levels within each treatment, we used an intercept-only binomial GLMM predicting bat performance, accounting for repeated measures. In this type of model, the parameter estimate for the intercept can be interpreted to determine if bats did better than random choice (after Maynard et al., 2019). We used one-tailed binomial tests to assess if individual bats performed better than chance (50%) during the two-choice trials.

To explore how bat strategies varied across trials, we tested if bat performance could be predicted by certain behavior patterns (such as movement speed, amount of distance traveled or trajectory shape). Search behavioral parameters were log-transformed where appropriate and histograms inspected for outliers before analysis to

meet assumptions of normality. We fitted the data to a GLMM with a binomial distribution pooling trials across all experimental treatments (excluding trials where tracking was unreliable due to bat flight or leaving the back of the arena). Fixed effects included average velocity (cm/s), distance travelled (cm), movement time (s), decision distance (cm), and path shape, with Bat ID as a random effect. To test the significance of each fixed effect as a predictor of bat performance, we used a model simplification approach (Crawley, 2013). No interactions were included in the models due to limited sample size. If a significant effect was detected in the model ($P < 0.05$), we used a post hoc Tukey contrast adjusted for multiple comparisons to examine any differences.

Behavioral strategies are also likely to be context dependent, and individuals can show plasticity in their strategies. To examine how bats may adjust their search behaviors as the difficulty of the task increases, we isolated the successful bat trials from banana and odor solution (concentrations 100% - 0.1%) treatments. We fitted linear mixed models (LMM) with treatment as an explanatory variable and different trajectory measures (average velocity, distance travelled, decision distance) as response variables (using restricted maximum likelihood, lme in package 'nlme'; Pinheiro et al., 2018). Bat ID was included in the model as a random effect to account for repeated testing of individuals.

Finally, we investigated the role of head scanning in bat localization strategies by quantifying head movements during the successful trials when the bats were localizing banana and odor solution treatments. We used a GLMM with a Poisson distribution to test if there was an effect of either treatment or path shape on the frequency of head

scanning events and used a likelihood ratio test to compare a null model to the fitted model separately for each variable. To test if bats changed their head scanning behavior with distance from the odor source, we compared the average number of head scanning events for each bat that occurred before and after the bat made a decision using a paired Wilcoxon sign-ranked test.

All analyses were carried out using R (version 3.5.0, R Core Team, 2018) and RStudio (R Studio Team, 2016).

3.3. Results

We recorded 648 behavioral assay trials across 10 individual bats and seven experimental treatments. Bats made a choice (correct or incorrect) in 529 trials. Due to limitations in the field, the number of trials for each treatment for each bat was not equal. The minimum number of trials recorded for a treatment was five and the maximum number of trials for a treatment was 19. All 10 bats were tested across all experimental treatments except for three individuals, who were not exposed to the odor-only treatment.

Average temperature was fairly consistent across all trials (27.9 C ± 0.04 SE), and did not have a significant effect on bat performance (all trials pooled, binomial GLMM, $z = 1.414$, $P = 0.158$). Average relative humidity varied slightly more across trials (70.4% ± 0.11 SE) and did have a significant effect on bat performance (all trials pooled, binomial GLMM, $z = -2.032$, $P = 0.042$). To account for this variation, average relative humidity was included as a random effect in the generalized linear mixed models. There was also no effect of trial order on performance; that is bats were not

more successful at localizing odors in later trials than trials early in the experiment (all trials pooled, binomial GLMM, $z = -1.491$, $P = 0.136$).

3.3.1. Experiment 1: Localization of food reward using odor

In this experiment, we established whether bats could consistently and successfully locate a rewarded odor. Bats were reliably able to locate the location of a rewarded odor, with eight out of ten individuals performing better than chance in a two-choice assay (one-tailed binomial test, $P < 0.05$, n = 10 bats, 120 trials, 8 – 18 trials per bat). For the two bats that did not perform better than chance, they only made a choice during three (Bat 7) and six (Bat 8) out of ten trials, suggesting low motivation and not lack of tracking ability. On average, bats successfully located the odor reward 90.7% (± 6.99 SE) of the time (excluding trials where bats did not make a choice), exhibiting non-random preference for the odor-rewards side (intercept-only binomial GLM, $P < 0.01$).

3.3.2. Experiment 2: Role of acoustic cues during reward localization

In the first part of this experiment (Experiment 2A), we tested whether bats would localize an attractive odor cue without the appropriately matching echolocation cue. On average, bats performed better than chance at locating the odor-rewarded side, even when there was not an accompanying shape cue (intercept-only binomial GLM, $P < 0.01$) and successfully chose the odor cue in most of the trials (79.7% ± 8.24 SE, n = 10 bats, 72 trials, 4 – 9 trials per bat). In the second part of the experiment (Experiment 2B), we tested if bats could successfully locate an odor cue when no salient echolocation cues were present, by removing shape cues (i.e. banana piece or cosmetic sponge). Again, bats performed better than chance at locating the odor-rewarded side in both

treatments (intercept-only binomial GLM, $P < 0.01$ for both treatments). The average success rate for bats localizing an odor without a distinctive echolocation target was lowest compared to other experimental treatments (76% ± 5.72 SE, n = 7 bats, 64 trials, 3 – 11 trials per bat). Comparing bat performance across treatments from Experiments 1 and 2, experimental treatment had an effect on localization success across all 10 bats (binomial GLMM, $F = 3.5308$, df = 2). Bats were more successful at locating the banana reward compared to trials when there were no distinctive echolocation cues available to guide them ($z = -2.652$, $P = 0.0217$) (Figure 3.3A). Neither start latency (time at top of the arena before moving downward) or decision distance had an effect on bat performance.

3.3.3. Experiment 3: Effect of odor strength on localization success

We also tested how decreasing odor concentrations would affect bat olfactory localization performance. Overall, bats performed better than chance when locating 100%, 10% and 1% odor concentrations (intercept-only GLMM, $P < 0.01$ for all three treatments). Average percent success decreased with a decrease in concentration and bats had the highest average success rate when localizing the 10% odor solution (79.57% ± 4.73 SE). Bats were least successful when searching for the 0.1% odor solution, particularly when compared to the 10% ($z = 2.838$ $P = 0.0233$, Figure 3.3B). While four out of 10 bats performed better than predicted by chance at locating the 10% concentrations (binomial one-tailed test, $P < 0.05$), we did not have sufficient power to

make conclusions on individual performance due to limited trial sample sizes for most individuals (3 – 11 trials per bat, per treatment after 'no-choice' trials were removed).

3.3.4. Behavior and Movement Analysis

Across all experiments, we analyzed bat movements to quantify and categorize

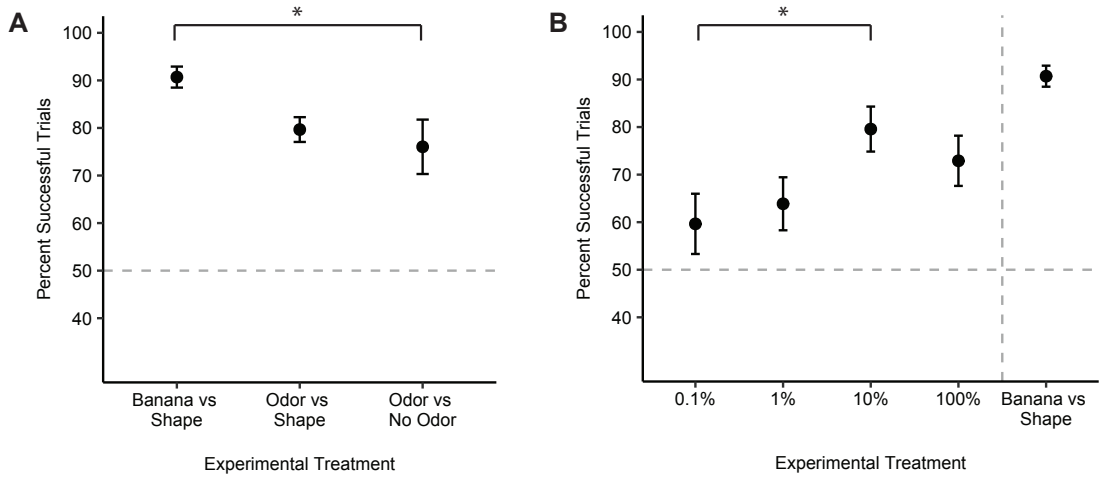

Figure 3.3. A) Comparison of the average success rate (percent of trials where bat correctly chose S+) across treatments for Experiments 1 and 2. Dots and bars indicate mean ± s.e.m. Bats performed better than expected by chance in all three treatments (intercept-only GLMM, $P < 0.05$). Asterisks indicate a significant difference in performance between treatments (binomial GLMM with repeated measures, $P < 0.05$). B) Comparison of the average success rate across treatments for Experiment 3 (percent banana concentration). Dots and bars indicate mean ± s.e.m. Bats performed better than expected by chance when localizing 1%, 10%, and 100% banana concentrations (intercept-only GLMM, $P < 0.05$). Asterisks indicate a significant difference in performance between treatments (binomial GLMM with repeated measures, $P < 0.05$). The results of the banana treatment were not included in the analyses of this experiment, but are included in this graph as a reference. The dashed horizontal line indicates chance levels of success (50%) in both A and B.

the potential odor localization strategies bats are using to localize an odor source. Only trials where bats crawled along the back of the arena to reach their choice (S+ or S-) were included in this analysis (n = 420 trials), consisting of 79% of all recorded trials in which bats made a choice (420/529 trials). Of the analyzed trajectories, 53.1% of

trajectories were from trials where the odor was presented on the left side of the arena (223/420 trials) and 46.9% were trials where the odor was presented on the right (197/420 trials).

We log-transformed average velocity and total distance travelled to meet assumptions of normality. Inspection of the distribution for decision distance revealed a bimodal distribution (Figure 3.4A). When separated between successful and unsuccessful bat trials, there was a peak in number of successful trials where bats made their decision between 25 and 35 cm from the stimulus (Figure 3.4B,C). This bimodality was not seen when looking only at unsuccessful trials (Figure 3.4B). Neither distance travelled nor average velocity had a significant effect on bat performance, but there was a significant relationship between bat performance and trajectory shape (GLMM, F = 4.067, df = 3).

Looking only at trials where bats successfully located the banana odors, there was a significant difference in the log-distance travelled during the trial, log-average velocity and decision distance across treatments (excluding treatments from Experiment 2, n = 327 trials) (LMM, $P < 0.05$). Bats travelled a shorter distance when localizing the 10% odor concentration compared to 1% (z = -3.411, P = 0.005) and banana (z = 2.86, P = 0.034) treatments. Bat trajectories were also more direct (as measured by straightness) when localizing 10% compared to 1% (z = 3.083, P = 0.0176) and banana (z = -3.016, P = 0.0214). Bats also moved fastest when navigating towards the 10% odor, particularly when compared to the banana treatment (z = -2.753, P = 0.046). Decision distance was

variable across treatments, with bats making their final decision closer to the banana stimuli compared to the 100% concentration (z = -2.988, P = 0.0214).

All four locomotor patterns were observed in successful trials across treatments, but *bottom casting* was significantly more frequent in successful bats than in unsuccessful bats (z = 2.688, $P < 0.01$) (Figure 3.2B). All individuals used each of the four search strategies at least once. To validate our qualitative categorization of search strategy, we compared the total distance traveled, path straightness, and decision distance using a repeated-measures ANOVA (with Bat ID as a random factor). Straightness was significantly different between path shapes (F = 21.09, $P < 0.001$, Figure 3.2D). Both *direct* and *middling* strategies were significantly straighter than either *casting* strategy (Tukey's pairwise comparison, $P < 0.001$) but were not significantly different from each other (t = -1.183, P = 0.634). Similarly, straightness of *top casting* and *bottom casting* did not differ significantly from each other (t = 1.143, P = 0.660). However, bats did travel significantly further (total distance) when using the *top casting* strategy compared to all other strategies ($P < 0.05$ in pairwise comparisons, Figure 3.2C). The decision distance was not significantly different between *top casting* and *direct* strategies (t = -2.041, P = 0.171), but both were significantly farther away from the correct stimuli compared to the other two strategies (pairwise comparison, $P <$ 0.001, Figure 3.2E). Based on these differences, we conclude that the strategies are qualitatively and quantitatively different from each other. The *top casting* strategy is characterized by the farthest traveled distance, the furthest decision distance and least straight trajectory compared to the other three strategies. While similar to *top casting* in

straightness and total traveled distance, *bottom casting* had the closest decision distance of all four strategies. In contrast, the *direct* strategy was the straightest path observed in these trials and bats made their decision at similar distances compared to *top casting*. While similar to the *direct* strategy in straightness and total distance travelled, the decision distance for the *middling* strategy was closer to the correct stimuli, but not as close as in *bottom casting* trajectories.

To analyze head scanning behavior, we pooled successful trials from which we were able to obtain high quality reconstructions of their trajectories from Experiment 1 (banana) and Experiment 3 (percent odor concentrations), resulting in a total of 247 trials across 10 individuals (15 – 40 trials per individual). We observed 849 total head scanning events across all trials. Most head scanning behavior occurred at distances between 60 and 80 cm from the odor source; i.e., when the bats were at the top of the arena (Figure 3.5A). Bats performed significantly more head scans both before starting their downward trajectory towards the odor source (Wilcoxon sign-rank test, $V = 53$, $P = 0.005$) and before making their final direction decision (Figure 3.5B, Wilcoxon sign-rank test, $V = 55$, $P = 0.001$). Neither concentration or path shape had an effect on the total number of observed head scanning events (GLMM likelihood ratio test, $P > 0.05$).

3.3.5. Estimating the Odor Concentration Gradient

Odors in the arena were not evenly distributed, but the odor structure in the arena was consistent with a Gaussian distribution with the highest concentrations recorded immediately above and next to the odor stimulus. The odor concentrations declined rapidly with distance from odor in both horizontal and vertical directions (Figure 3.4D).

After five minutes (the maximum trial time), odor concentrations along the vertical axis stayed either constant or increased, staying higher along the middle of the arena compared to the horizontal odor distribution. Along the horizontal axis, the odor concentration gradient dropped close to 0 (or below detectable levels using the PID) around 30 cm from the odor source, while it did not drop to 0 until between 35 to 55 cm in the vertical direction (Figure 3.4E).

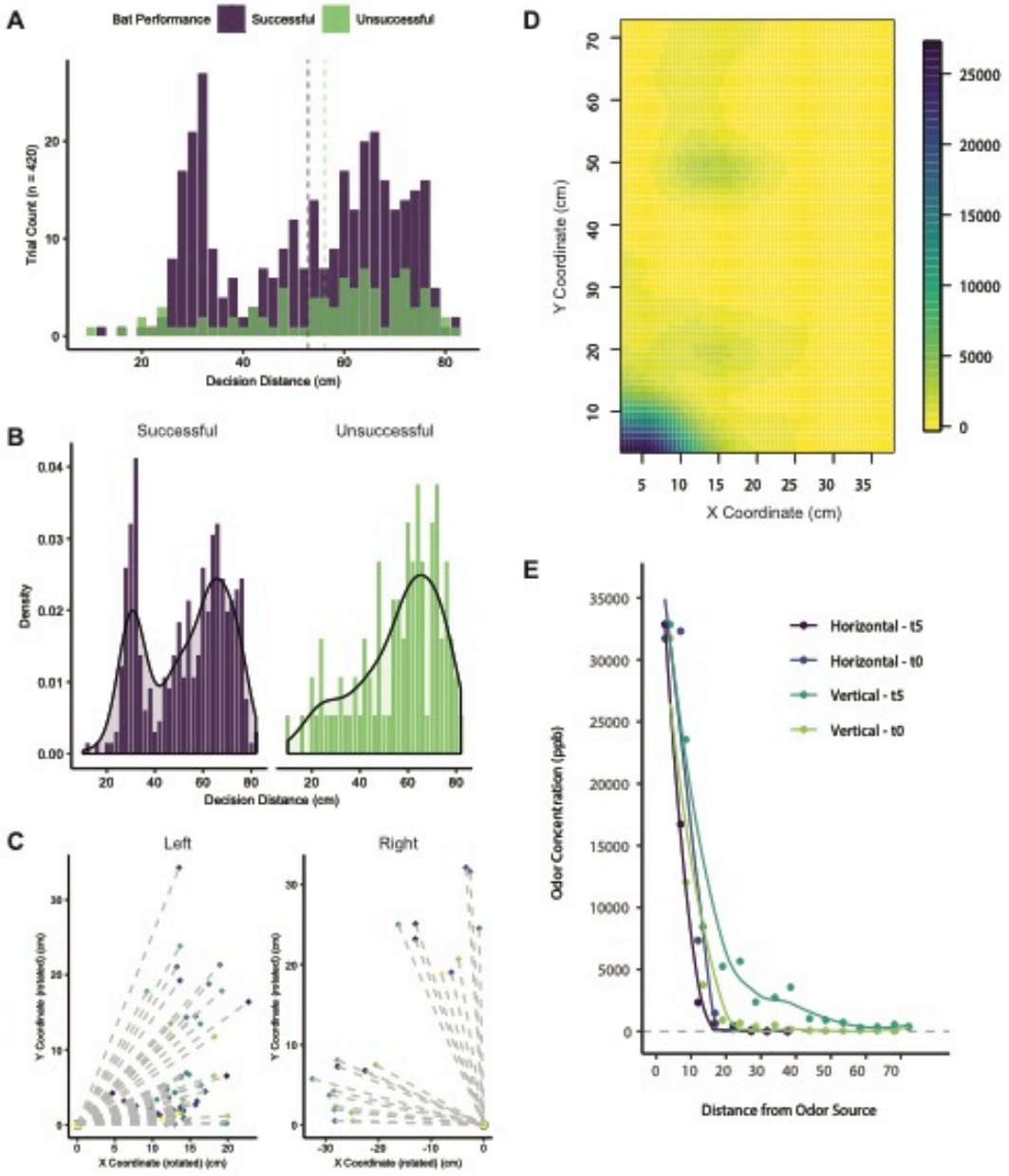

Figure 3.4. A) Histogram showing the distribution of decision distances across trials, shown for both successful and unsuccessful trials (trials pooled across all experimental treatments and individuals, n = 420 trials). Dotted vertical lines represent the mean decision distance for each category (successful, unsuccessful). B) Histograms comparing distribution of decision distances, normalized by density for successful trials (purple, n = 327 trials) and unsuccessful trials (green, n = 93 trials).

Figure 3.4 (cont). C) Graphical plot of the pooled trials with decision distances (diamond) within 25 – 35 cm of the odor stimulus (square). Different colors represent trials from different individuals. Left side represents trials where the odor stimulus was on the left side of the arena (n = 60 trials), and right side when the odor stimulus was on the right side (n = 21 trials). Coordinates were obtained from EthoVision XT 13, then rotated and transformed to standardize the location of the odor stimulus (based on a Cartesian coordinate system). D) Heat map with a Gaussian smoothing function (smooth.2d, theta = 4 in package 'fields', Nychka et al., 2017) representing the measured concentrations (in parts per billion) measured within the arena when 0.1 ml 100% banana extract was placed on the left side. E) Plots showing the decay of odor concentration of 100% banana extract (in parts per billion) with distance from the odor source along the horizontal (purple) and vertical (green) axes of the behavioral arena both immediately after odor placement and 5 minutes after odor placement.

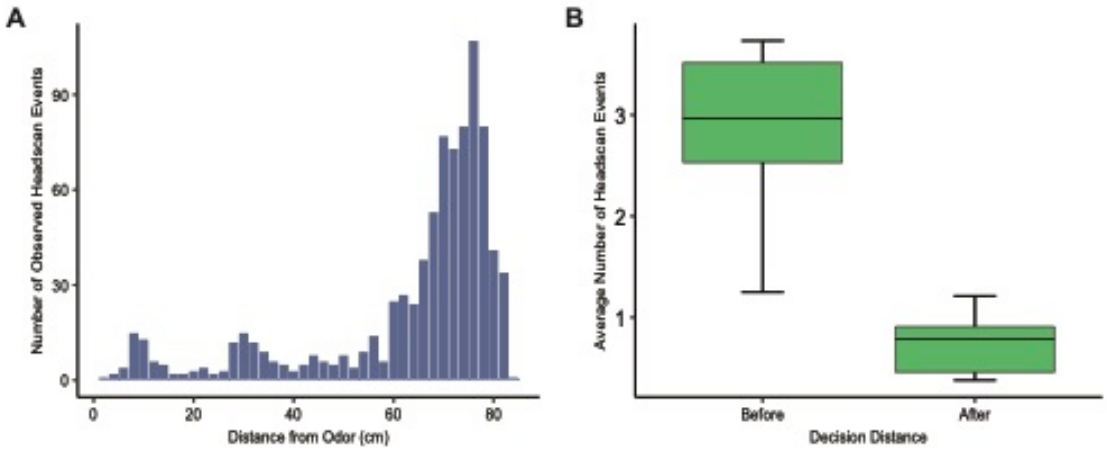

Figure 3.5. A) Histogram showing the distribution of number of individual head scanning events (n = 849 events) relative to distance from odor source, pooled across all successful trials and individuals for Experiment 1 and Experiment 3 (10 bats, 247 trials). B) Boxplot of the average number of head scanning events (average of all trials for each individual (n = 10) observed before and after bats made their final change of direction in successful trials Experiment 1 and Experiment 3. There is a significant difference in average head scanning behaviors before and after bats reach the decision distance (paired Wilcoxon sign-rank test, $P < 0.01$).

3.4. Discussion

Our results suggest that bats use klinotactic olfactory tracking strategies similar to other terrestrial mammals, including humans (Jinn, 2019), mice (Gire et al., 2016; Liu et al., 2020), and rats (Bhattacharyya and Bhalla, 2015). While previous work demonstrated that bats are able to detect and discriminate concentration gradients to localize odors rewards (Laska, 1990a; Laska, 1990b), this is the first study to specifically quantify the locomotor patterns and olfactory search strategies of bats. Similar to previous research demonstrating the importance of olfactory cues in other echolocating and non-echolocating bat species (Hodgkison et al., 2007; Korine and Kalko, 2005; Parolin et al., 2015; Tang et al., 2007; Thies et al., 1998; von Helversen et al., 2000), northern yellow-shouldered bats were able to localize an odor reward using olfaction under experimental conditions that controlled for echolocation cues. By recording the bats movements in an open-field type behavioral setup (as opposed to a Y-maze or other choice paradigm), we were able to exploit this behavior and quantitatively describe the search routes bats followed while localizing an odor reward. We show that bats were able to find odor sources even when the measured concentration of odors in the air was very low, consistent with previous studies on bat olfactory sensitivity (Laska, 1990a) which reported detection thresholds in the range of approximately 3-15 parts per billion.

Olfactory localization strategies are often multi-modal, with animals integrating olfactory cues with visual, mechanosensory and acoustic inputs (Cardé and Willis, 2008; Gomez-Marin et al., 2010; Vickers, 2000). While bats, including Neotropical leaf-nosed bats, use vision as part of their orientation and foraging strategy (Gutierrez et al., 2014),

it is unlikely that visual cues provide much detailed information. Most bat-dispersed fruits in the Neotropics do not change color with ripening, opposite of the pattern observed in many bird- and primate-dispersed plant species (Kalko et al., 1996; Lomáscolo et al., 2010). Visual cues and acoustic cues are also less reliable against cluttered backgrounds (such as a fruit cluster on a leafy branch), and it has been shown that removal of visual cues does not significantly impact bat foraging success (Korine and Kalko, 2005; Thies et al., 1998).

Like other Neotropical leaf-nosed frugivores, *Sturnira* produces low intensity, high frequency echolocation calls, with peak frequencies ranging from 65 kHz to 92 kHz (Jennings et al., 2004; Yoh et al., 2020) emitted via the nose. Fruit and nectar feeding bats within the family Phyllostomidae (including *Sturnira*) are thought to primarily use echolocation for general orientation, as well as final approach and selection of food items (Gonzalez-Terrazas et al., 2016b; Kalko and Condon, 1998; Leiser-Miller et al., 2020; Thies et al., 1998). We controlled for potential effects of echo-acoustic information during odor localization in Experiment 2. Bats performed better than expected by chance even when the echolocation cue was paired with the non-rewarded, no odor control (Experiment 2A) and there was no obvious echolocation cue (Experiment 2B, Figure 3.3A). Based on these results, we conclude that acoustic cues did not significantly contribute to the bats ability to discriminate the odorized targets and that that the primary sensory cue bats were using in these assays was olfaction. This is further supported by observations that even when bats chose the wrong side (S-), they did not attempt to consume the control sponge, which would be predicted to have the

same acoustic signature at the S+ sponge, while they often bit and tasted the banana-scented sponge.

We observed a peak in decision distance at 25 – 35 cm from the odor source for successful attempts across all concentrations. This distance coincided with an inflection in the steepness of the odor gradient which provided optimal conditions for bats to detect spatial differences and orient towards the higher concentrations. At distances where the odor is detectable, but the concentration gradient is still shallow, large movements or changes in direction (casting) are more efficient (Catania, 2013). Once the gradient becomes steeper near the source, short movements, head scanning, and bilateral inputs may be sufficient to find an odor source (see for example Figure 7 in Catania, 2013; Jinn, 2019). That this distance is also about the same as the observed olfactory decision distance of mice following an odor plume (Liu et al., 2020) suggests this may be a common pattern across mammals.

Our trajectory analysis identified four distinctive search locomotor patterns routinely displayed by all bats within the experimental chamber (Figure 3.2A). Since bats are also expected to perform this task in flight at high velocities, we anticipated the possibility of exaggerated or unusual locomotor patterns relative to terrestrial mammals such as dogs or rodents. Contrary to expectations, none of the recorded tracks exhibited the forward zig-zag pattern that characterizes the olfactory tracking trajectories displayed by walking mammals or flying insects (Svensson et al., 2014; Vickers, 2000). The least common pattern, top casting, had broad lateral movements back and forth across the top of the arena that could be characterized as zig-zagging, but these zig-zag

motions rarely resulted in net forward motion. This type of movement at the edges of a concentration gradient are consistent with the model posed by Catania (2013), with large movements and serial sampling helping to provide directional information in shallow gradients. The most commonly observed successful locomotor pattern was the direct strategy, representing a relatively direct search pattern with no major changes in orientation during the track (Figure 3.3A). Assuming bats are receiving motivational cues from the top of the arena, then this strategy could be compared to the "aim and shoot" strategy used by some flying insects to locate odor sources (Cardé and Willis, 2008), which does not always result in a successful search, similar to what we observed in our experiments. This downward movement can be paired with serial sampling as observed in other taxa (Catania, 2013; Liu et al., 2020), allowing the animal to more accurately reassess the direction of the odor gradient when they get nearer the odor source (Jinn, 2019; Thesen et al., 1993). This behavioral strategy is also consistent with the middling locomotor pattern we observed, wherein the bats moved down the center of the chamber until they had sufficient directional information within the odor gradient to select the correct direction.

Bats may be able to pair movement with increased active sampling, such as sniffing (Baker et al., 2018; Khan et al., 2012; Vergassola et al., 2007) and simultaneous head-scanning (Gomez-Marin et al., 2010; Khan et al., 2012). Sniffing and head scanning improve the efficiency of klinotactic olfactory localization by allowing an organism to maintain its body orientation within an odor plume, while permitting a longer period to sense and integrate the chemical signal (Dusenbery, 1992). Liu et al.,

(2020) proposed that at distances far from the source serial sampling (sniffing) is performed with whole body movements, which may be replaced by increased head scanning as mice approach the odor source. In contrast, the bats in our study performed most of their head scanning movements at the top of the arena before moving towards the odor source (Figure 3.5A) and were only observed when bats were stationary. As these bats use echolocation for orientation (Hernández-Canchola et al., 2020) and bats are known to use head movements to keep biosonar beam projections fixated on obstacles and targets (Surlykke et al., 2009), we were not able to separate head movements associated with sniffing from those associated with biosonar emissions. It remains possible that bats process olfactory inputs during passive breathing and echolocating (Eiting et al., 2014; Wachowiak, 2011) but the predominance of biosonar for navigation may preempt the use of head scanning purely for olfactory search. Although more research in this area is needed, this observation represents a key departure from the current synthesis of olfactory search models proposed for mammals (Baker et al., 2018; Catania, 2013; Liu et al., 2020). This, in combination with having narrow nostrils for emitting pulses through the nose suggests that bats may be constrained in their ability to use stereo-olfaction and head scanning during the final approach phase of olfactory searches.

Trial-and-error or route-following strategies could help bats overcome the trade-offs between echolocation and serial sampling. Of the four locomotor patterns observed, bottom casting appeared to be consistent with what has been termed route-following in other animals. This strategy consisted of rapidly approaching one of the targets and

coming within several centimeters of S- before sharply changing direction towards the S+, which suggests the bats were following a route with a limited number of known options. Under natural foraging conditions, animals supplement sensory information such as olfactory cues with long-range navigation and cognitive strategies. Studies in rats have demonstrated that under certain circumstances (e.g., small number of targets and known locations), strategies such as route-following are faster and more robust than gradient following or casting (Bhattacharyya and Bhalla, 2015; Gire et al., 2016), particularly as familiarity with the task increases (Gire et al., 2016). In our assay, there were only two possible locations for the odor reward, which with experience shifts the olfactory task from "where" to "which" (Bhattacharyya and Bhalla, 2015). Bats, particularly nectar feeding bats, have been shown to have extraordinary spatial working memories (Henry and Stoner, 2011; Toelch et al., 2008; Winter and Stich, 2005). Short-tailed fruit bats (*Carollia*) rely more strongly on spatial memory than sensory cues when foraging in the wild (Fleming et al., 1977) and spatial memory may even overshadow the use of sensory cues such as odors (Carter et al., 2010). Bat flight is also metabolically expensive, so relying on spatial memory and returning to quality foraging locations may be more efficient for foraging fruit bats than following odor plumes, provided they are exploring a known space.

Flying bats are exposed to highly variable olfactory environments when foraging under natural conditions, but they also use olfaction while crawling in roosts or when perched in trees, where their movements are slow and the local olfactory landscape is more stable. Our results suggest that when bats are restricted to crawling, they displayed

olfactory tracking strategies similar to other terrestrial mammals, with only minor constraints arising from echolocation. Future work quantifying how bats navigate towards an odor source while flying would provide more insight into how bats use odors in their natural environment, as well as how use of olfactory sensory cues integrates with other navigational strategies such as echolocation and spatial memory.

4. FLYING BATS RELY ON SERIAL SAMPLING TO LOCATE ODOR SOURCES

4.1. Introduction

Chemical cues and signals are commonly used by animals for detecting and locating resources via olfaction. Finding the source of an attractive odor is a complex task, dependent on nasal anatomy and physiology, sensory integration, and complex movement patterns (search strategies). Neurophysiological processing includes the integration of olfactory stimuli with spatial and temporal memory cues (Baker et al., 2018; Rinberg et al., 2006). Animals can also take advantage of the spatial and temporal gradients of odors in the environment to localize their source by comparing the intensity or timing of odors (Gardiner and Atema, 2010; Takasaki et al., 2012) they move through the environment (klinotactic olfactory search). Animals that navigate to odors sources while flying are exposed to even more complex fluid environments, which can affect the ability to detect odor gradients or resolve fluid movement direction (Vickers, 2000). Animals' olfactory search strategies are thus shaped by the speed of chemical signal transduction and speed of information processing relative to their own speed and maneuverability.

Like in other sensory modalities, there appears to be a speed-accuracy trade-off in olfactory decision-making. Mice demonstrate an increase in accuracy with an increase in odorant exposure time when asked to perform an olfactory discrimination task (Abraham et al., 2004; Rinberg et al., 2006) and require more time as the olfactory task becomes harder (Rinberg et al., 2006). When tracking in turbulent environments, many

animals will reduce their travel speed and move in undulating patterns in order to increase their chance of detecting an odor plume (Moore et al., 1991; Thesen et al., 1993). This reduction in travel speed likely allows animals to adjust their sampling and maximize odorant exposure, with sampling rates (sniffing) also increasing as animals slow down (Hirsch, 2010; Thesen et al., 1993). Flying animals are less flexible in their ability to slow down or pause while following an odor plume. While male moths are well known for their ability to follow pheromone plumes to locate females (Cardé, 2016), relatively faster flying insects such as tsetse flies don't make wind and plume adjustments in flight (Griffiths et al., 1995), instead relying on a "aim-then-shoot" strategy (Cardé and Willis, 2008). Little is known about how flying vertebrates compensate for the speed problem when tracking odors in flight. With the exception of some seabirds and vultures (Grigg et al., 2017; Nevitt et al., 2008; Stager, 1964), birds are unlikely to use long-distance olfactory cues to locate odor sources due to reduced olfactory morphology and gene repertoires (Bird et al., 2018; Yohe et al., 2020). Bats, particularly fruit and nectar-feeding species, are known to use olfactory cues while foraging (Korine and Kalko, 2005; Thies et al., 1998). While fruit-eating bats are highly sensitive to some fruit-typical odor compounds (Laska, 1990a), studies on bat olfactory receptor genomes and histological analysis suggest bats likely display intermediate olfactory capabilities compared to other mammals (Barton et al., 1995; Eiting et al., 2014; Hayden et al., 2010; Yohe et al., 2020). Given the constraint of olfactory processing at normal flight speeds, behavioral mechanisms would be important for coping with during flight.

Airflow rate and sampling time play a role in odorant sensitivity and discrimination in mammals. Inhalation of air is required to access olfactory receptor neurons within the mammalian nose, as these neurons are not activated when an odorant is simply blown at the nose (Wachowiak, 2011; Wesson et al., 2008b). Increased sampling rates (such as sniffing) may help animals resolve increasingly difficult olfactory discrimination tasks (Wesson et al., 2008a) or compensate for loss of sensitivity while moving. Unlike other mammals, most bats rely on echolocation for navigation, which imposes its own requirements on respiration in addition to support olfaction. Neotropical leaf-nosed bat (Family: Phyllostomidae) echolocate primarily via nasal emission, likely as an adaptation to permit navigation via echolocation when carrying fruit by mouth to a feeding roost. Nasal echolocation may preclude bats from maximizing olfactory inputs because olfactory behaviors such as sniffing probably cannot interrupt or supersede the timing of pulse emissions, although it is still possible that the intervening inspirations could be sufficient to support odor deposition and olfactory processing (Eiting et al., 2014).

Here, we studied the foraging and flight behaviors of Jamaican fruit-eating bats (*Artibeus jamaicensis*) locating food rewards using only odor cues, and quantitatively describe the olfactory foraging strategies of bats in flight. Jamaican fruit-eating bats are medium sized frugivores (29 -51 g) and are common in a variety of habitats throughout Central and South America (Ortega and Castro-Arellano, 2001). They are frugivorous and feed on a variety of fruits, including figs (*Ficus*), pepper (*Piper*), and banana (*Musa*) (Handley and Leigh, 1991; Ortega and Castro-Arellano, 2001). Jamaican fruit-eating

bats have relatively larger olfactory bulbs and thicker nasal epithelium than insect-eating bats, suggesting use of olfactory cues during foraging (Bhatnagar and Kallen, 1974a) and demonstrate preferences for odor cues in behavioral experiments (Hodgkison et al., 2013; Parolin et al., 2019). As a result, these bats likely attend to olfactory cues during foraging.

We presented wild-caught individuals with either an odor reward (banana) or odor stimulus (banana emulsion) randomly located upon one of five potential options in a flight cage. We used synchronized video recordings from two cameras to reconstruct the bat flight paths in three-dimensional space. We first confirmed that bats could successfully locate the correct platform based only on olfactory information, and then used the resulting three-dimensional trajectories to characterize what strategies the bats used to locate odors in flight. If bats are able to detect and use the olfactory gradients from a distance to solve an olfactory localization task, we expected that individuals would sample the entire air space and then directly approach the odor reward perch first more often than predicted by chance. We also predicted that flight paths would display patterns observed in other flying animals, such as exaggerated zig-zag patterns used by insects or the spiral search pattern displayed by vultures (Baker, 1990; Stager, 1964). Alternatively, bats may instead rely on cognitive or memory-based learning strategies to locate the odor location. For example, captive owl monkeys did not perform above random chance at locating food rewards using sensory cues, but appeared to instead show preferences for certain locations and followed consistent routes between feeding boxes (da Costa and Bicca-Marques, 2014). Similar serial-sampling or route following

behavior has been observed in rats and mice. During early trials searching for reward via olfaction, mice relied on odor gradients, but as familiarity with the task increased they would instead sample each possible location sequentially (serial sampling; Gire et al., 2016). If bats instead rely on a serial sampling or memory-based strategy, we would predict that bats would not perform better than random chance at approaching the correct platform first and instead visit each platform sequentially before making a choice.

4.2. Methods

4.2.1. Animal Capture and Care

Behavioral experiments were conducted from September 8 – December 8, 2019, at the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07'14.5" N, 79°42'08.2" W). Bats were captured using mist nets set over streams and across flyways in Soberania National Park, Panama. Following capture, bats were sexed, weighed, and aged by examining the epiphyseal-diaphyseal fusion of the fingers (Brunet-Rossinni and Wilkinson, 2009), and only adult, non-reproductive individuals of *A. jamaicensis* were kept for experiments. For the first 24 hours, bats were held in a mesh cage and provided water and banana *ad libitum*. To facilitate association and to train bats to retrieve banana from a wooden platform, the banana in the mesh cage was placed on a single platform in the center of the cage.

On the following night, individual bats were released into the experimental chamber (5 x 5 x 2.5 m outdoor flight cage) for up to 30 minutes to acclimatize to the flight chamber. Bats were offered banana pieces placed on wooden platforms and those individuals that spontaneously removed and consumed banana from the platforms during

this trial period were identified and retained for behavioral experiments, resulting in a total of N = 36 bats (male = 20, female = 16). Tested bats were individually marked with hair-trimming patterns. These patterns allowed individual identification during experiments. Hair trimming patterns lasted long enough to support recognition if bats were subsequently recaptured, allowing us to avoid re-testing of individuals over the study period, although recaptures were rare (one individual over the course of the study period). Following behavioral trials, bats were released at their initial capture location.

4.2.2. Ethical Note

Animal capture was approved by the Panamanian authorities (Authoridad Nacional de Ambiente, ANAM permit number 2017-0102-2020-A36). All experiments were conducted according to protocols approached by the Institutional Animal Care and Use Committee at Texas A&M University (AUP 2017-0139) and the Smithsonian Tropical Research Institute Animal Care and Use Committee (SE/AP-22-19).

4.2.3. Experimental Setup

We measured olfactory localization behavior in flying bats using a multiple-choice assay with standard operant procedures. Experiments took place between 19:00 and 05:00 hours and were synchronously recorded using two Basler Ace model ac640-um digital video cameras mounted on tripods, connected to a desktop computer running Media Recorder 3.0 (Noldus Technology, United States). We ran all experiments in complete darkness to minimize visual cues during the experiments. Cameras were placed in opposite corners of the room, oriented to maximize coverage of the space. The experimental chamber was illuminated using infrared LED light strips hung from the

ceiling, with supplemental lighting from two infrared LED spotlights. Five wooden platforms (1.2 m high) were placed evenly across the back of the experimental chamber (70 cm from the back wall of the chamber). Platforms were spaced 90 cm apart from each other, with the end platforms placed 70 cm from the side walls (Figure 4.1). The top of each platform was a 15.2 x 15.2 cm square, large and stable enough for the animals to land on them, and the platforms were covered in clear vinyl to allow for easy and thorough cleaning between trials. Stimuli were positioned in the center of each platform. This setup was same for bat acclimation and experimental trials.

Bats were held together in a soft mesh cage (60.9 x 60.9 x 91.4) in a dark quiet location between trials. We randomized the order that individuals were tested each night. The location of the reward (S+), which was either banana or banana-scented target, was pseudo-randomized in each trial to ensure that it was placed on each platform at least once, and its position was not repeated consecutively between trials. Although experiments are presented and analyzed separately, the trials for both treatments were randomized within and across experimental nights, to reduce potential confounding effects of learning and to ensure sufficient motivation for individuals (by providing banana trials every 3rd or 4th trial). The sides of the experimental chamber were covered in heavy, black cloth to minimize airflow from outside. We measured the temperature and relative humidity within the flight cage at the start and end of each trial.

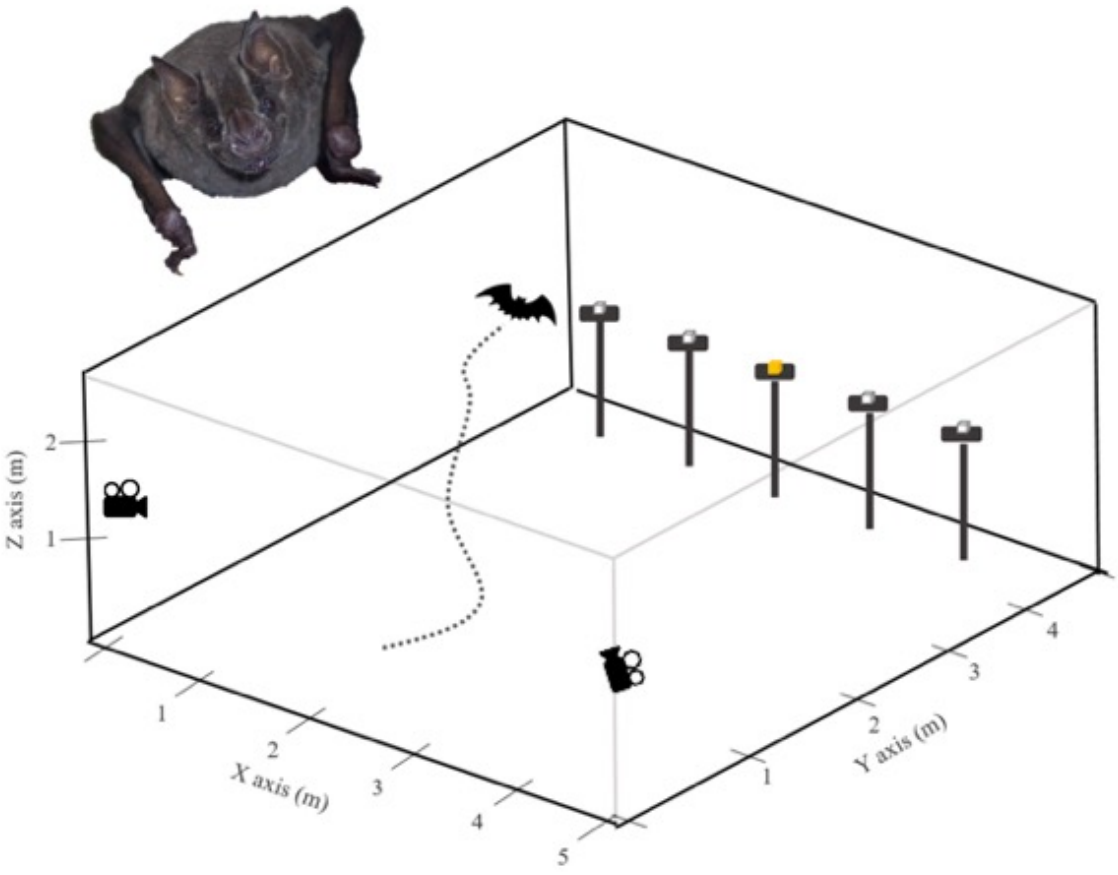

Figure 4.1. Diagram of the experimental arena, showing the position of the cameras and stimulus platforms. Individual bats (*Artibeus jamaicensis*, inset) were released into the room opposite the row of stimulus platforms. Cubes on the platform represent the cosmetic sponges used to present the odor and control stimuli. Yellow indicates location of the odor stimulus. Bat photograph by A.F. Brokaw.

4.2.3.1. Experiment 1: Localization of food reward using odor

In this experiment, we confirmed that bats would be able to successfully localize an odor reward in the experimental arena. During each trial, bats had the option to choose between a banana reward (S+) and control objects (S-). One banana piece (approximately 2.5 x 2.5 x 1.5 cm) was placed on a random platform in the experimental

chamber. The other four platforms held controls. The control object (S-) were cosmetic sponges cut into the same shape as the banana piece and were soaked in water prior to experiments. We supplemented the banana piece with 0.1 ml of 100% food-grade banana baking emulsion (LorAnn Professional Kitchen, Michigan, USA). Stimuli were prepared and placed immediately prior to the start of the behavioral trial. The trial was started when a bat was released from one side of the room, approximately 4 m from the row of platforms. Trials lasted until the bat landed on the platform containing the piece of banana, or after a maximum of 20 minutes had elapsed. If bats did not attempt to locate or feed on the banana after 20 minutes, the trial was ended and a "no-choice" result was logged.

4.2.3.2. Experiment 2: Role of acoustic cues in reward localization

It is possible that bats would be able to distinguish the banana pieces from control objects based on echolocation, since echoes reflected off of banana pieces and banana-shaped sponge pieces might contain discriminable differences in acoustic features. In this experiment, we tested if bats could successfully locate the odor (S+) when all five stimuli were the same sponge material. Instead of banana, an odor cue was prepared by soaking a cosmetic sponge cut in the same shape as the banana in a banana-sugar mixture. The banana mixture was prepared by blending 10 grams of ripe banana with 10 grams of 30% w/w sugar in water solution. The odor cue sponges were then supplemented with 0.1 ml of 100% banana baking emulsion, the same as the banana pieces in the previous experiment. Controls were cosmetic sponges cut the same as the odor stimuli and soaked in water. Trials began when the bat was released into the

experimental chamber and continued until the bat landed on the platform containing the odor cue, or after 20 minutes had elapsed. If bats did not attempt to select any target after 20 minutes, then a "no-choice" result was logged.

4.2.4. Behavior Scoring and Analysis

We determined that the bats had made a choice when they landed directly on the top of the platform or landed on the side and crawled to the top of the platform to access the banana or sponge. Trials were scored a 'success' when bats correctly chose the platform containing S+. If bats performed this landing behavior on one of the platforms holding a control, it was scored as an unsuccessful trial, but bats were allowed to continue the trial until they landed on the correct platform, or until a total of 20 minutes had elapsed. Additionally, we scored the investigatory behavior of bats over the course of the entire trial. Investigatory behaviors were assigned to one of two categories: 1) inspection behaviors, where the bat flew near and above the platform and lowered its head, directing its nose towards the platform, or 2) approaching behaviors, when bats flew directly towards the platform, then changed direction to fly away. For each trial, we recorded the total number of investigation events across all five platforms. We also counted the number of unique platform investigations performed by each bat. For example, if a bat investigated platform A three times and platform B two times, then the total number of investigation events would be five, and the number of unique investigations would be two. The final choice, defined by landing, was not included as an investigation event. We also recorded the time and location of the first platform investigated and time to and location of first choice (landing). Bat behavior was

manually scored using either EthoVision XT 13 (Noldus Technology, United States) or BORIS 7.9.16 (Friard and Gamba, 2016). All manual behavior scoring was carried out by the same individual (A.F.B).

In light of preliminary evidence that flying bats investigated several platforms before making a final choice, we recognized the need to establish a set of objective criteria for defining an "olfactory investigation" event (or approach) as distinct from routine flight maneuvers. These events were distinguishable based on measurements of instantaneous flight speeds, decelerations, body movements, and head orientations when approaching the platform as well as how closely the bats got to the target. To do this, we reconstructed the three-dimensional flight paths for a subset of trials in Experiment 2, using the Track3D module of EthoVision XT 13. From the three-dimensional reconstructions, we compared the flight speed of the bat during an inspection behavior to the flight speed (m/s) when the bat was flying across the room not near the platforms. The three-dimensional reconstruction provides the position of the bat in the x, y and z dimensions, measured in centimeters. We used the distance in the z dimension during an inspection behavior to calculate a minimum distance at which bats investigated a platform, based on the known height of the platforms (1.2 meters). To minimize the effects of potential tracking errors in our data when comparing speeds and estimating vertical distance from the platform, we only analyzed time points where the intersection error of the sample in the three-dimensional track was less than 10 (intersection error is the distance between two lines in the 3D space connecting each camera to the tracked object).

4.2.5. Statistical Analysis

Performance for each experiment was calculated as the percentage of trials in which the bat correctly chose the platform containing the odor stimuli (S+). Trials where bats did not land on a platform (no-choice) were excluded from analysis. We used one-tailed binomial tests to assess if each individual bat performed better than chance (20%, or 1/5) during the trials. During trials, we observed that bats frequently investigated platforms via inspection or approach behaviors. If bats are following an odor plume in order to select the correct platform, then we would predict that their first investigation of a platform would be the location of the odor stimulus. Therefore, we used one-tailed binomial tests to assess if individuals performed better than chance (20%) in their first inspection or approach to a platform. If bats were not following an odor plume, but instead using odor to make their final choice, we would predict that the last platform investigated would be the same as their final choice. We used one-tailed binomial tests to assess if individuals inspected the correct platform immediately before making their final choice.

If bats were using information in an odor plume to locate the odor rewards, we would predict them to have investigated significantly fewer than half of the platforms on average before making a successful decision. Looking only at trials where bats successfully chose the odor cue (by landing on it), we examined the distribution of total number of investigation events (sum of both inspection and approach behaviors) and number of unique platform investigations. To test if there was a common number of unique platform investigations, we used a repeated-measures ANOVA (‘lme’ in package

'nlme', Pinheiro et al. 2018) to compare the number of trials successful bats explored before making their final choice, setting number of investigated platforms as a categorical variable (0 – 5 unique platforms investigated). In this categorization, 0 would represent a trial where the bat performed no investigation behaviors, immediately landing on the correct platform, while 5 would indicate a trial where the bat approached all 5 platforms at least once. We compared between variables using Tukey's post-hoc comparisons, adjusted for multiple comparisons ('glht' in package 'multcomp', Hothorn et al., 2008).

From the data obtained in Experiment 2, we compared the average speed (m/s) of bats when investigating the platforms to the average speed of 'control' events in the same flight path. Pseudo-random 'control' speeds were manually selected from times when the bat was flying straight across the arena (not changing direction or landing). Speed was compared between investigation and control flight time points using paired t-tests (with each individual data point representing an average of points from across the trials).

All analyses were carried out using R (R Core Team, 2018) and RStudio (R Studio Team, 2016). Three-dimensional flight paths were visualized using Plotly (Sievert, 2018).

4.3. Results

We recorded a total of 487 trials from 36 individuals (16 females and 20 males) across both experiments (banana and odor cue) from September 30 – November 27, 2019. Environmental conditions in the experimental arena was relatively consistent

across the study period. Temperatures ranged from 24.2 to 26.2 C (25.33 ± 0.03 standard error of measurement (s.e.m)) with relative humidity ranging from 57% to 90% (85.2% ± 0.27 s.e.m.).

4.3.1. Experiment 1: Localization of food reward using odor

In Experiment 1, we confirmed that bats could successfully locate the platform holding a banana reward using olfactory cues. We recorded 301 trials across the 36 individual bats (16 females and 20 males). The minimum and maximum number of trials recorded per bat was 5 and 12, respectively. Bats made a choice in 253 of the trials (84% of the trials). A 'no-choice' was logged for an average of 1 trial per bat (and ranged between 0 and 5 trials). For further analysis, we removed individuals who made a choice in less than 5 trials, resulting in a total of 253 trials across 34 individual bats (15 female, 19 male). On average, bats successfully located the odor reward in 88.2% of trials (excluding trials where bats did not make a choice) (Figure 4.2). Individually, all bats demonstrated a success rate for locating the banana reward significantly more often than expected by chance (one-tailed binomial test, $P < 0.05$).

4.3.2. Experiment 2: Role of acoustic cues in reward localization

In Experiment 2, we controlled for possible echolocation cues by presenting bats with 5 identical stimuli, only one of which was scented with banana odor. Using some of the same individuals as tested in Experiment 1, we recorded 182 trials across 25 individuals (12 female and 13 male). Bats made a choice in 153 of the trials (84.0% of the trials). Two individuals made a choice in less than 5 of the recorded trials and were excluded from further analyses, resulting in 23 individuals (12 female and 11 male). On

average, bats successfully located the odor reward in 87.4% of trials (excluding trials where bats did not make a choice, Figure 4.2), and all but two individuals demonstrated a success rate significantly higher than expected by chance (one-tailed binomial test, $P < 0.05$).

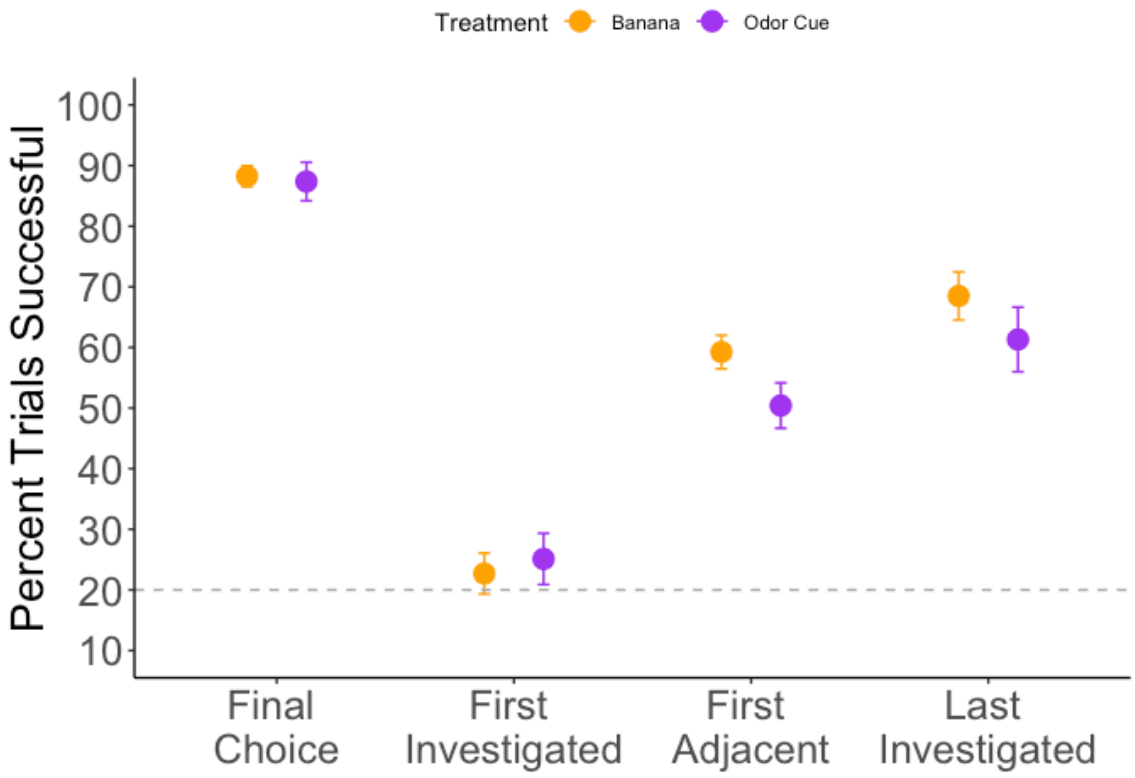

Figure 4.2. Average percent of successful trials for bats' final choice (landing on the platform), first platform investigated, investigating the adjacent platforms, and last platform investigated in Experiment 1 (banana) and Experiment 2 (odor cue). Success is defined as landing on or approaching the platform containing the odor stimulus, except for first adjacent, which includes investigating the platform(s) immediately adjacent to the platform containing the odor stimulus.

4.3.3. Olfactory Search Strategies

The results for Experiments 1 and 2 confirm that bats are able to use odors to correctly identify an odor reward. If bats are using odor plumes to locate the source of an attractive odor, we would predict that they would be more likely to approach those platforms early in the search. However, we found that the first platform bats investigated in either experiment was rarely the platform containing the odor (first investigation successful: Banana 22.35% ± 3.37 s.e.m and Odor Cue 25.1% ± 4.22 s.e.m), and bats did not do better than random chance at first investigating the platform with the odor cue in either experiment (one-tailed binomial tests, $P > 0.05$). We then considered that bats may be able to use the shape of the odor plumes to narrow down the general location based on odor but might not be able to pinpoint exactly which platform holds the reward. If we included investigations of platforms adjacent to the platform holding S+, bats were slightly more successful (Banana 59.2% ± 2.76 s.e.m. and Odor Cue 50.4% ± 3.74 s.e.m.). Even if bats were not using olfactory cues to locate the odor-scented platform, they may use olfactory information to assess presence or absence of the odor during these investigation events. We then looked at the location that bats investigated last (before landing on a platform). The location of last bat inspections and final choices matched in 67.8% of banana trials and 61.3% of odor cue trials (Figure 4.2).

Looking only at trials where bats correctly found the odor cue (Experiment 2, n = 133 trials across 26 individuals), we measured two different type of investigation events: 1) inspection flights and 2) approach flights (Figure 4.3A). Bats investigated (sum of both inspection and approach flights) all platforms about six times before making a

decision (weighted average = 6.23) (Figure 4.3B). Bats tended to inspect more platforms than they approached (Figure 4.3C). Looking at the number of unique investigations, bats inspected or approached about three of the five platforms at least once before making a decision (weighted average = 2.95). The number of trials in which bats uniquely investigated between zero and five platforms differed significantly (repeated measures ANOVA, $F = 10.57$, $P < 0.001$) (Figure 4.3D). Specifically, bats investigated either three or four platforms before making a decision significantly more often than investigating zero, one, two, or five platforms (adjusted pairwise comparisons, $P < 0.05$).

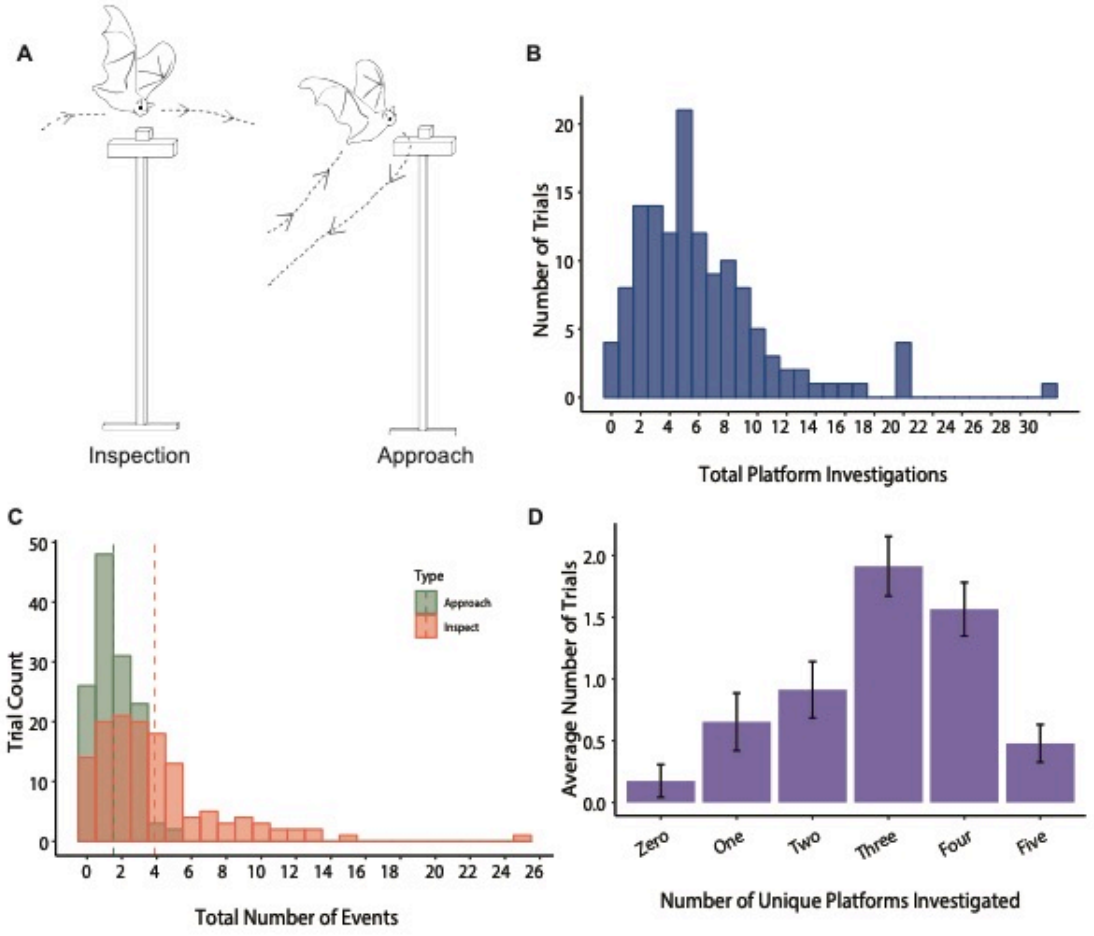

Figure 4.3. A) Diagram of observed investigation behaviors during experimental trials. B) Histogram for the total number of platforms investigated by bats successfully locating an odor cue (Experiment 2). C) Histogram for number of each type of investigation event (inspection and approach) observed across successful trials (Experiment 2). D) Comparison of the number of unique platforms investigated by bats across successful trials in Experiment 2. Bats investigated three and four different platforms significantly more often than investigating zero, one, two or five platforms (repeated-measured ANOVA, $P < 0.001$). Error bars indicate within-subject standard error.

We reconstructed the flight paths of 40 successful trials from nine individuals from Experiment 2, ranging between two and eight trials per bat (Figure 4.4A). Bats moved significantly slower when inspecting the platforms compared to 'control' points across trials (paired t-test, $t = 24.847$, $P < 0.001$, Figure 4.4B-C). During these

inspection events, bats were close to the top of the platform, averaging an estimated minimum vertical distance of 5.8 cm (± 0.66 cm), with most inspection events occurring when the bat was a minimum of 10 centimeters above the platform (Figure 4.4D). This measurement does not account for bat distance from the platform in the x or y direction, and so is likely an underestimate of bat distance from the top of the platform (and presentation sponge).

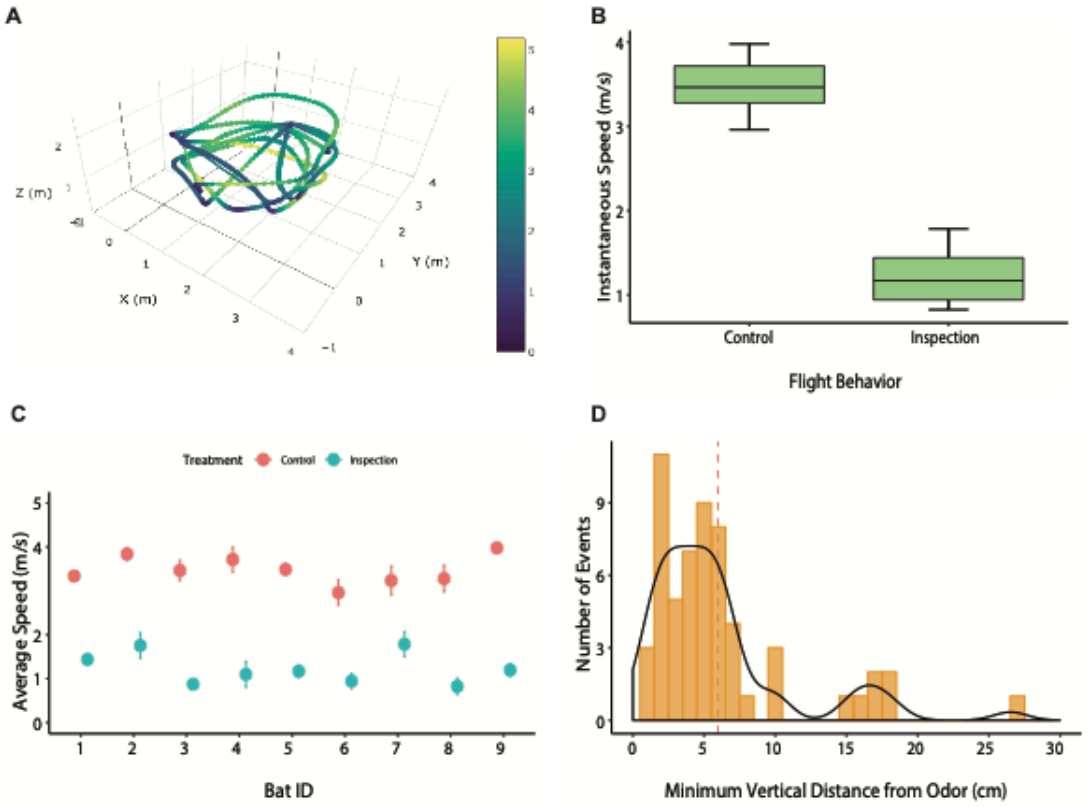

Figure 4.4. A) Example three-dimensional flight path reconstruction of a bat navigating to the odor cue (on the middle platform). In this trial, the bat flew over and approached the middle platform (holding the odor cue) several times before landing. Color scale represents bat speed in meters per second. B) Average flight speed for 'control' timepoints and during 'inspection' events. Bats moved significantly slower during 'inspection' events (paired t-test, $P < 0.01$). C) Flight speeds for 'inspection' and 'control' timepoints, averaged for each individual bat. D) Histogram of vertical distance

from top of the platform for all inspection events.

4.4. Discussion

Olfactory cues play an important role in food evaluation and selection of many fruit- and nectar-feeding bats (Korine and Kalko, 2005; Parolin et al., 2015; Sánchez et al., 2006; Thies et al., 1998; von Helversen et al., 2000). Our results in flying Jamaican fruit-eating bats suggest that while bats appear to use odor as a foraging cue, it is unlikely that they are exploiting odor plumes or airborne olfactory information to locate the source of attractive odors. Instead, bats in our assay appeared to use a serial-sampling search strategy, in which they investigated potential locations at random, then using olfactory cues to make their final selections. This cognitive rather than sensory strategy may be an adaptation to compensate for decreased olfactory inputs in these fast-flying vertebrates.

If bats were following an odor plume or gradient to locate the source of an attractive odor, we would expect that bats would first investigate the platform containing the odor source, or a platform nearby. Instead, we found that bats appear to randomly approach their first platform, first investigating the correct platform only 20-30% of the time (Figure 4.2). On average, bats inspected about half of the available platforms before making a final selection, clearly indicating that they could not isolate the source without closely inspecting the platforms first. While our experimental arena presented an artificial foraging environment, this behavior appears consistent with observations of natural foraging behavior in *A. jamaicensis*. On Barro Colorado Island in Panama, Jamaican fruit-eating bats were observed spending time ‘scouting’ out fruit trees within

their home ranges and would then return to the same tree up to eight nights in a row (Morrison, 1978a). Several species of *Artibeus* specialize on *Ficus* figs. Individual *Ficus* trees ripen asynchronously, so at any given time point, only one or two trees may hold edible fruit. However, during this ripening, *Ficus* trees produce an abundance of fruit over multiple days, thus acting as a steady resource over a short time frame (Bonaccorso and Gush, 1987) and providing a predictable food source in the short term. Field studies in both Panama and Costa Rica suggests that Jamaican fruit-eating bats make consistent use of flyways during foraging (Heithaus et al., 1975; Morrison, 1978a). *Artibeus jamaicensis* appears to separate commuting and searching behaviors, following flyways to areas with known fruiting trees. This contrasts with the behavior of a sympatric fruit-eating species *Carollia perspicillata,* which is instead on constant 'alert' for potential feeding locations (Fleming et al., 1977). *Carollia* specialize on the fruits of Neotropical pepper plants (*Piper*) (Cloutier and Thomas, 1992), which produce fruit for very short periods of time and are much more ephemeral compared to *Ficus.*

Under challenging tracking conditions, other organisms will adjust their locomotor patterns to increase their ability to detect and locate an odor source. Dogs will decrease their speed up to 50% when deciding the direction of an odor trail, while also increasing their sniffing rates (Thesen et al., 1993) and will adjust their posture and speed depending on distance from an odor trail (Jinn et al., 2020). Other animals adopt pause-travel behaviors, in which they stop movement and increase sampling or sniffing (Hirsch, 2010; Togunov et al., 2017). Based on the dynamics of flight, bats and other flying vertebrates are limited in their ability to slow down or reduce movement while

flying. Unlike some nectar-feeding bats, fruit bats such as *Artibeus* are unable to perform sustained hovering flights, due to large body size and constraints of wing morphology (Struhsaker, 1961), and bats generally take fruit from branches in flight, rarely hovering in front to pick them up (Leiser-Miller et al., 2020; Thies et al., 1998). Since odor plumes at distances greater than several centimeters become much more unpredictable and stochastic, it would be difficult for bats to reduce speeds and detect plume edges, as observed in flying insects navigating in an odor plume (Cardé and Willis, 2008; Vickers, 2000). We observed that during platform inspections, bats significantly reduced their speeds when flying over the platforms, which they could do without intending to land (Figure 4.4B). The reduced speeds were achieved by changing wing beat patterns and carefully executed aerial maneuvers that produced brief spinning pauses just above the platforms before gliding away. In addition to reduced speeds, bats also frequently directed their nose downwards when near the platforms. This behavior is consistent with previous observations in which bats would perform exploration flights followed by multiple approaches to a selected fruit before final fruit acquisition (Korine and Kalko, 2005; Thies et al., 1998). Many Neotropical fruit bats (including *A. jamaicensis*) are maneuverable fliers, with broad wings and low aspect ratios that are well suited for navigating in dense forest clutter (Marinello and Bernard, 2014; Norberg and Rayner, 1987). In addition to enabling bats to navigate through cluttered areas, this maneuverability also allows bats the opportunity to closely approach and examine potential food items using both olfaction and echolocation.

Echolocation may impose a strong constraint of bat olfactory sampling during flight. Echolocation is linked to the respiratory and wing-beat cycle, with echolocation pulses generally emitted during expiration (Falk et al., 2015; Suthers et al., 1972). Observations from preliminary recordings of bats flying in our experimental arena suggested that bats did echolocate throughout the course of the olfactory task. Fruit-eating bats such as *Artibeus* produce high frequency, frequency modulated calls of low amplitude (Korine and Kalko, 2005; Yoh et al., 2020) from their nose. Bats were observed to change their echolocation call patterns as they approach a potential target but did not display a distinct terminal phase before selecting a fruit (Korine and Kalko, 2005; Thies et al., 1998), and these calls facilitate the location of an individual fruit within a branch. It is still unclear how nasal airflow associated with echolocation could affect odorant deposition and absorption on the olfactory epithelium, although possible variation in the way air flows through the olfactory recess may help maximize odorant deposition (Eiting et al., 2014).

In Experiment 2, we controlled for the use of echolocation cues to distinguish the rewarded from non-reward stimuli and found that bats still successfully located the banana-scented platform. During measured inspection events, bats were very close to the odor stimulus (frequently less than 10 centimeters from the top of the platform, Figure 4.4D). While bats were certainly using biosonar to inspect the platform and sponge from farther away, the acoustic cues presented during Experiment 2 should be nearly identical, differing only in the presence of an odor cue. Bats may only be able to clearly discern the olfactory cue from within this shorter range (Chapter 3), particularly at the

higher speeds associated with flight. I propose that bats rely on spatial memory and echolocation to orient within the experimental area, and then use a combination of echolocation (to resolve presence of an object) and olfaction to make a final decision. This strategy differs slightly from previously proposed scenarios, in which odors are a cue used for detection of potential food resources, and echolocation is used to precisely localize a selected fruit at close range (Korine and Kalko, 2005; Thies et al., 1998). Similar behaviors have been observed in nectar feeding bats, where more background clutter facilitated greater use of odor cues to select rewarded flowers (Muchhala and Serrano, 2015).

Various factors can affect the strength and distribution of an odor plume in space. The amounts of odor presented in this experiment were relatively small, especially in comparison to the odor plume from a tree with hundreds to thousands of fruits on it. Search for resources can occur at large scales (between feeding patches) to small scales (within a feeding patch). Rather than representing discrete foraging locations, our experimental setup may more closely mimic multiple branches of the same tree, on which some individual fruits are ripe while others are not. Having located the potential food source, bats are then able to use olfactory cues to discriminate between individual options. At this smaller scale, it is also possible that the odors emanating from the stimulus platform would overlap in space with nearby platforms. If bats are able to detect the general location of the odor, but not discriminate fine-scale plume structure, then they might first approach an adjacent platform as they narrow down the odor location. However, even when accounting for potential general localization, bats still

only approached the correct general area about 50% of the time. Previous work using the same olfactory stimulus found that the odor concentration rapidly decreased, dropping below detectable levels by around 30 cm (Chapter 3), which is less than the distance between platforms in this experiment. While we attempted to reduce airflow in the experimental arena, airflow due to bat flight movements could disrupt odor plumes, thus reducing the predictability of an odor gradient as a reliable orientation mechanism.

As canopy frugivores, Jamaican fruit-eating bats are generally not food-limited and can return to the same location several nights in a row without depleting the number of available fruit (Bonaccorso and Gush, 1987). Spatial memory has a strong effect on fruit bat and may even overshadow sensory cues such as odors or acoustics (Carter et al., 2010). A similar shift in strategy to spatial cues has been observed in mice (Gire et al., 2016) and rats (Bhattacharyya and Bhalla, 2015), with animals relying on using a serial sampling strategy based on spatial memory to locate the rewarded odor source. Since bats in flight are generally moving faster than mice or other terrestrial animals, and the location of individual trees does not change, quickly sampling a few potential locations and using odor to make a final selection may be more efficient than trying to follow unpredictable odor plumes, particularly in a cluttered environment. Canopy-feeding frugivorous bats may also be more vulnerable to predation, as suggested by strong lunar phobia (Morrison, 1978b), and so moving quickly while searching for resources may also be safer.

Overall, our results suggest that Jamaican fruit-eating bats rely primarily on cognitive strategies for locating potential resources, although sensory cues such as

olfaction and echolocation likely play an important role in the discrimination and selection of fruits. Although this may be the result of the small-scale nature of our experimental setup, this is likely also the result of adaptive behaviors to maximize foraging efficiency at larger scales, supported by field observations in this and other fruit-eating species (Fleming et al., 1977; Morrison, 1978a; Villalobos-Chaves et al., 2017). Bats may use olfactory and visual cues to supplement their spatial maps and can learn of new resources via independent exploratory flights or social learning from the mother or other conspecifics (Harten et al., 2020; Ratcliffe and ter Hofstede, 2005). Future work could take advantage of advances in tracking technology to further examine how bats integrate sensory information while foraging. Understanding how bats locate resources on the landscape is important for predicting how changes in landscape features (such as habitat loss or fragmentation) may affect bat populations, particularly in tropical forest ecosystems (Bianconi et al., 2007; Bianconi et al., 2012).

5. SUMMARY AND CONCLUSIONS

Bats have been longtime models for exploring the neuroethology of the vertebrate auditory system, including specializations in support of active sensing through biosonar and acoustic communication, but the morphology and behaviors associated with their other sensory systems (i.e,. vision, somatosensorial, olfaction) have received far less attention. Behavioral observations and empirical studies confirmed the importance of olfaction for some types of foraging bats, but no studies have addressed how bats track olfactory information to locate the source of an attractive odor. This is a significant gap because the most widely-accepted current models of olfactory tracking by vertebrates (Catania, 2013; Liu et al., 2020) rely heavily upon behavioral strategies that simply do not translate well to the bat's aerial ecological niche. Although work in pheromone following in flying insects has provided a steady foundation for characterizing strategies flying animals may use to track odor plumes (Cardé, 2016; Cardé and Willis, 2008; Svensson et al., 2014), flying insects still move relatively slowly within their olfactory landscape, whereas bats are mammals with more sophisticated olfactory systems moving at significantly higher speeds through larger and more complex spaces. Similarly, work on odor tracking in mammals has been limited to terrestrial and subterranean species, which operate in vastly different odor environments (Baker et al., 2018). The goal of this dissertation was to address this gap by characterizing the comparative morphology and olfactory search strategies of these diverse, fast moving and flying mammals.

To approach this, my thesis utilized three different approaches: First I tested the hypothesis that nostril separation may enhance the use of tropotactic olfactory tracking mechanisms by comparing the external nasal morphology of bats species differing in diet, foraging habitat, and echolocation strategies. This hypothesis was based on observations comparing morphology across many vertebrate taxa (Stoddart 1979) but had only been tested in one group of chordates (sharks; Kajima 2005). Opposite of my predictions, I found that bats observed using olfaction for foraging (fruit and nectar-feeding bats) had narrower nostrils, which might be a result of their reliance on nasal echolocation. Secondly, using two- and three-dimensional tracking software, I then quantified the olfactory search behaviors of fruit-eating crawling bats. This experiment fills a critical gap because it allowed me to directly compare how bats executed an olfactory search behavior to the results obtained from terrestrial mammals tested under essentially identical conditions. The results revealed both similarities and differences that may reflect constraints arising from biosonar demands. Thirdly, I investigated how flying bats locate a reward following only olfactory cues to address the effects of flight speeds and biomechanics on search strategies. While crawling bats were able to track the odor concentration gradient in patterns similar to terrestrial mammals, I found that flying bats relied more heavily on memory-guided route-following strategies when locating an odor in flight. Based on my observations of head-scanning in crawling bats and the discovery of stereotyped olfactory investigation maneuvers commonly displayed by flying bats, it appears clear that echolocation imposes a significant and complex constraints on odor plume following in bats, despite also contributing important spatial

cues to the overall search strategy. In summary, the results of this thesis provide compelling evidence that 1) echolocation has constrained the evolution of the olfactory substrate in bats, 2) bats are capable of recognizing and exploiting olfactory gradients to efficiently locate odor sources, and 3) that once an attractive odor is detected, bats compensate for the added challenges of speed and echolocation by first employing memory-guided behaviors to locate potential resources, followed by olfaction and other sensory cues once they are near enough to the target to exploit strategies similar to land-based mammals.

5.1. Trade-offs between Olfaction and Echolocation

It is very difficult to disentangle respiration, olfaction, and echolocation in bats, particularly in bats that emit echolocation pulses from their nose. The narrow nostril widths observed in nasal emitting bats almost certainly dominates the evolution of nostril morphology in phyllostomid bats and thereby imposes a critical constraint on the potential for tropotaxis. Bat echolocation is highly directional, with sound focused in a narrow beam projection pattern in the forward direction, creating a "field of view" in front of the bat (Jakobsen et al. 2013). In addition to frequency, echolocation beam shape depends on the size and shape of the emitter (i.e., the distance between nostrils and size/shape of the nostrils and nose-leaf; Hartley and Suthers, 1987; Pye, 1988). Movements of the noseleaf during echolocation appears to allow the bat to adjust vertical directionality (Vanderelst et al., 2010) and are used to induce time-variant manipulations in the acoustic properties of succeeding pulses to improve target localization and classification (Zhang et al., 2020). In addition to the phyllostomids,

some vespertilionid bats are also thought to facultatively use nasal echolocation (Arbour et al., 2019; Seibert et al., 2015), mainly as a way to maximize sensory input from the environment while avoiding detection from potential prey (tympanate moths). Seibert et al. (2015) observed that these nasal emitting species tended to have more upturned nostrils than oral emitting species, further supporting a possible relationship between nare morphology and nasal echolocation.

If bats are limited in their ability to employ tropotactic tracking strategies, they may still be able to take advantage of spatial distributions of odors by performing casting behaviors. Casting behaviors involve the side-to-side motion, either of the entire body (such as in flying moths, Cardé, 2016) or of the nose (as observed in rodents, Khan et al., 2012; Liu et al., 2020). I found that crawling bats rarely displayed casting behaviors when navigating towards an odor reward. Furthermore, while bats were observed using head scanning behaviors, the pattern of this head-casting behavior was opposite observed in other mammals. Based on olfactory tracking models such as the one proposed by Catania (2013), it is predicted that mammals would rely more on bilateral cues at close proximities to the odor source, where concentration gradients are the steepest. Relatedly, animals would also increase head movements and sniffing to resolve these gradients at close distances (Liu et al., 2020). In contrast, in my experiments head scanning movements in bats were observed at the farthest distances from the odor source, and bats did not perform these movements while in motion. Since head movements are tightly linked and perhaps even guided by the directionality of biosonar emissions, the primary use of echolocation for navigation may also constrain the use of

these casting mechanisms. It was previously demonstrated that the bat's "acoustic gaze" as measured by head direction is intractably linked to its flight motor output by a neural transformation of biosonar auditory cues automatically transformed into head and flight motor commands (Ghose and Moss, 2006); thus, bat head scanning movements are subservient to its biosonar behavior, which supersedes the use of head scanning for olfactory purposes.

The relationship between echolocation and respiration may also limit the use of sniffing behaviors, which are frequently observed in mammals and are thought to improve olfactory detection and processing. During flight, bat respiratory rhythms are biomechanically linked to wingbeat rhythms (Speakman and Racey, 1991) and while bats can emit variable numbers of biosonar pulses during a single expiration, there is no evidence that they can modify respiratory timing or volumes independent of wingbeats. Sniffing behaviors modulate the rate of airflow over the olfactory epithelium, which influences the sorption of odorants (Oka et al., 2009), but for bats to engage in sniffing they would need to have volitional control of inspiratory timing and force independent of flight biomechanics, which based on available evidence seems unlikely. Higher flow rates can result in a greater total absorption of odor molecules, but also produce less relative absorption (a smaller fraction of suspended odorants is able to be absorbed; Eiting et al., 2014). The relationship between airflow rate and absorption is also dependent on the type of odorant and spatial arrangement of olfactory receptors within the nose. For example, strongly-sorbed odorants are removed from the air stream as they pass through the nasal cavity at low rates, and so do not activate receptors farther in the

airflow path (Wachowiak, 2011). Both humans and rats modulate their sniffing behavior during olfactory tracking, by increasing their sniffing rate (Khan et al., 2012; Porter et al., 2007) or length of each sniff (Jinn, 2019). Although sniffing behavior is frequently observed in crawling bats, both in the context of stationary foraging and social interactions (e.g., Bartonička et al., 2010; Muñoz-Romo et al., 2011; Ramakers et al., 2016), it is unclear how much they might sniff while moving – either crawling or in flight. Physical and behavioral manipulations, such as temporarily blocking one or both nostrils, are not practical in bats since they rely so heavily on echolocation for general sensing of their environment. Advancements in three-dimensional models and simulations (Eiting et al., 2014; Vanderelst et al., 2010) may have promise for trying to disentangle the relationships between airflow, nasal morphology, and echolocation.

5.2. Strategies in Crawling and Flying Bats

Bats offer a unique opportunity to compare how a relatively large, complex organism navigates the olfactory environment under vastly different conditions (i.e., terrestrial versus in flight). I found that crawling bats navigated towards odor rewards using relatively direct paths, with some evidence for route-following. Crawling bats are able to resolve an olfactory gradient similar to other mammals, particularly rodents (Bhattacharyya and Bhalla, 2015; Gire et al., 2016; Liu et al., 2020), but I found no evidence that they used directional olfactory cues when searching in flight. I propose that this shift in strategy is due to challenges associated with flight speed and increased demands on the respiratory system via flight and echolocation. Similar differences were observed when comparing the pheromone tracking of walking and flying moths, with

walking moths orienting towards the odor source in nearly straight lines compared to the counter-turns and zigzag movements observed when flying (Willis and Baker, 1987). These differences are thought to be due to differences in the sensory signals available in each modality, such as vision and mechanosensory inputs. Even in the small-scale space of the experimental flight cage, bats moved as much as 16 times faster when flying compared to crawling. Although bats appear able to decrease their speed when investigating potential odor sources, this overall difference in speed surely makes following an odor gradient more difficult, both by decreasing the amount of time for odorants to deposit on the olfactory epithelium and making it harder for bats to resolve edges of existing odor plumes. This is also a challenge faced by flying insects, although recent computational fluid modelling suggests that flies (*Drosophila*) can adjust aerodynamic performance to direct airflow and olfactory stimuli towards their primary olfactory organs (Li et al., 2018). Even with reduced speeds, it is unclear if bats could take advantage of similar maneuvers in flight, although bats do demonstrate greater flexibility in their abilities to manipulate and shape air vortices while flying compared to birds (Hedenström and Johansson, 2015).

Comparisons between the results of my crawling and flying assays should be interpreted with some caution, mainly because they were done in two different species of leaf-nosed bat. Although both species are frugivores, they do differ in specifics of diet, morphology, and general habitat use. Northern yellow-shouldered bats (*S. parvidens*) are smaller than Jamaican fruit-eating bats (*A. jamaicensis*) and have slightly higher relative wing loads, suggesting increased maneuverability in flight (Marinello and Bernard,

2014). Compared to *Artibeus, Sturnira* are not strict canopy feeders, foraging on fruits from both canopy trees and more understory plants (Bonaccorso and Gush, 1987). Unlike other species in the subfamily Stenodermatinae, *Sturnira* lack a tail membrane (Reid, 2009), which has been hypothesized to make them more adept at climbing and crawling along branches and through dense foliage while foraging (N. Simmons, personal communication). Less is known about the natural foraging behavior of *Sturnira* compared to *Artibeus*, although both are known to repeatedly follow established commuting routes between day roosts and preferred foraging areas (Mello et al., 2008) and they reliably return to the same foraging sites over the course of several nights (A. Brokaw, personal observation). This suggests that use of spatial memory and route-following are central components of their search strategies while foraging in this species.

Search and foraging in bats and other animals are complex and multi-sensory behaviors. Shifts from olfactory gradient following to memory-guided behaviors under certain conditions are not unique to bats. Mice have been shown to locate odor sources without casting (Bhattacharyya and Bhalla, 2015) and will shift to serial-sampling strategies when the number of potential locations is low (Gire et al., 2016). Even with stops at multiple potential locations, mice moved faster and were more efficient following experience with the experimental setup (Gire et al., 2016). In the case of many fruit bats, including *Artibeus,* food sources are spatially patchy, but temporally predictable and so fast movement between potential options may be more energetically efficient than rely on sensory cues such as olfaction. The use of cognitive maps for locating food resources is further supported by recent work in Egyptian fruit bats

(*Rousettus aegypticus*), which engage in goal-directed long straight flights between fruit trees (Harten et al., 2020; Toledo et al., 2020). These flights are consistent within individuals and are not associated with wind direction, as they would be if bats were using wind-borne olfactory cues for orientation (Harten et al., 2020). Nevertheless, these routes must be learned from some initial discovery that may have depended upon the detection and tracking of olfactory cues, and routes must adapt to accommodate seasonal changes in fruit availability. Bats following a well-established route may yet improve foraging efficiency by using olfaction to more quickly determine the presence or absence of fruit at one location before moving on to the next location.

Even if bats are not routinely following odor plumes to locate resources, olfactory cues are likely still useful for bats to discover new resource locations, possibly combined with social information (Prat and Yovel, 2020). Individual-based models of olfactory and social cues in Cory's shearwaters (*Calonectris borealis*) found that while olfactory cues provided the foraging advantages over a wide range of environmental conditions, combining olfactory information with social information (via local enhancement) best described the movement patterns used by the birds (Bastos et al., 2020). Many species of bats (including *Artibeus*) are thought to take advantage of social cues while foraging by eavesdropping on the echolocation and social calls of con- and heterospecifics (Egert-Berg et al., 2018; Gager, 2019). Bat roosts may also serve as information transfer centers of potential foraging resources, with social learning modulated by olfactory cues (Ramakers et al., 2016; Ratcliffe and ter Hofstede, 2005; Teague O'Mara et al., 2014). My results suggest that even though bats may not use

olfactory plumes to follow odors to their sources, olfactory cues still play a role in location and selection of food resources. How bats integrate olfactory and other sensory cues to maximize foraging efficiency (or if they even do) still remains to be seen.

5.3. Bats Olfactory Cues and Signals

Chemical cues and signaling play important roles in mammalian social interactions, including in bats. While this dissertation focused on the use of olfactory cues by foraging bats, the morphology and behaviors related to olfactory tracking are likely still relevant in social contexts. In comparing external nasal morphology, I found that free-tailed bats (Family: Molossidae) had the widest relative nostrils compared to other insectivores. Molossids are well known for their strong pheromonal odors for marking roosts, and free-tailed bats have the ability to discriminate odors from different sexes (Bouchard, 2001), individuals (Englert and Greene, 2009), and offspring (Gustin and McCracken, 1987). Many free-tailed bat species also show sexual dimorphism in the presence of glands and production of associated odors, specifically a gular-thoracic gland that is found seasonally on males (Keeley and Keeley, 2004; Reid, 2009). Males use these glands to scent mark females and roosting sites (Heideman et al., 1990; Keeley and Keeley, 2004) and the odors may serve as a sexually selected signal for mate choice or territory defense in these species. Thus, olfactory tracking may yet be important even at long distances for high-flying, migratory and insectivorous bats such as Mexican free-tailed bats (*Tadarida brasiliensis*).

Olfactory information may also be used as long-distance navigation or homing cues or locating of resources other than food. Several studies have tested the role of

olfactory cues in roost localization and selection. Social odors do not seem to play a large role in roost selection by insectivorous species (Brown et al., 2020; Ruczynski et al., 2007), although these studies are potentially confounded by other factors such as neophobia. These studies also focus on the use of odors for the discovery of new roosts and not the use of odors for reorienting towards a known roost location. Overall, homing and navigation in bats is not well understood, mainly due to the difficulties of tracking these small, nocturnal animals. Early displacement studies, which measured return to known roost locations following displacement, suggest the use of visual landmarks at distances beyond 13 km (Holland, 2007), with more recent studies confirming the importance of visual landmarks in shaping the cognitive map of Egyptian fruit bats (*R. aegypticus*) (Toledo et al., 2020; Tsoar et al., 2011). However, the authors point out that these studies do not necessarily rule out the use of olfactory cues for navigating by bats and are limited to one species (a non-migratory, frugivorous pteropodid with rudimentary echolocation).

Olfactory based navigation has been proposed as a sensory mechanism for homing and migratory navigation in birds (Walcott et al., 2018). This olfactory navigation hypothesis (Papi et al., 1972) is based on the principles that atmospheric odors are distributed along a gradient and birds learn the odors associated with their roost or nesting area. Studies taking advantage of GPS-tracking technology in seabirds have found at least partial support for this hypothesis, with true navigation in several species requiring intact olfactory nerves (Abolaffio et al., 2018; Gagliardo et al., 2013; Wikelski et al., 2015) and evidence that birds detect potential volatile cues during flight

(Safi et al., 2016; Zannoni et al., 2020). The sensory and environmental cues used by bats during migration as still unclear, with some limited evidence for magnetic cues (Holland et al., 2010; Tian et al., 2019). Thus, it remains to be tested if migratory bat species, such as Mexican free-tailed bats (which are well-known for their strong-smelling roosts and natal philopatry, citation), may be able to employ atmospheric odor cues during migration.

5.4. Olfactory Ecology and Implications for Conservation

Sensory cues, including olfaction, are critical for many ecological processes, including species recognition, habitat selection, foraging efficiency, and risk assessment. A greater understanding of how and under what conditions animals use sensory cues can inform both how human activities might impact animal behaviors via sensory pollutants (Dominoni et al., 2020) and how sensory cues might be used to influence animal behavior for the purpose of conservation and management (Friesen et al., 2017).

As diffuse but potentially long-distance cues, chemosensory signals can serve to attract animals to a given location, such as nesting or roosting sites (Friesen et al 2017). Fruit and nectar-feeding bats play important roles in tropical forests as pollinators and seed dispersers (Kunz et al., 2011). It has been proposed that olfactory cues, such as artificial fruit scents, may be useful in attracting seed-dispersing species to recently deforested areas (Bianconi et al., 2007; Mikich et al., 2003). Fruit essential odors appeared to attract multiple species of frugivorous bats to degraded areas within the Atlantic rainforest (Bianconi et al., 2012) and placement of commercial fruits (mangos and bananas) increased abundances of fruit-eating species in deforested areas of southern

Mexico (Preciado-Benítez et al., 2015). These studies recorded species-specific differences in attraction and foraging patterns, with some species attracted to only certain scent cues (Mikich et al., 2003) and more captures of species known to frequently move between forest fragments (Bianconi et al., 2007; Bianconi et al., 2012). My results in a small-scale foraging task suggests that at least some bats (*Artibeus*) search mainly via spatial and memory- guided cues, then use olfactory cues for final discrimination and selection. Understanding how bat search patterns interact with use of different sensory cues (including olfaction) can improve the efficiency of these types of forest restoration efforts by situating olfactory cues in places where bats may be more likely to find them.

The experiments presented here were limited to relatively small-spatial scales, which may not be completely representative of natural foraging behaviors. Advances in three-dimensional flight tracking (such as thruTracker; Corcoran and Hedrick, 2020) can offer an opportunity to track bat behaviors at intermediate scales, such as behaviors and maneuvers as they approach a fruiting tree. Miniaturization and increased advances in on-board technology (such as acoustic detectors, GPS, accelerometers, etc.) can also provide more fine scale detail about what cues bats use to orient and navigate at the landscape level (i.e., Harten et al., 2020; Toledo et al., 2020), and allow for eventual comparisons between bats in different habitats or different feeding strategies.

In conclusion, while I found that bats were able to follow odor gradients to their source, odor plume following may not be the most efficient or adaptive strategy for foraging bats to locate food resources. This may be partially due both the morphological

and physiological constraints imposed by echolocation. There is still much to learn about how bats integrate olfactory cues with other sensory and cognitive modalities for resource selection and localization. Instead of using zigzag or casting behaviors at the edges of an odor gradient, bats using head scanning behaviors to orient and make an initial direction decision. They then use short-range (within 20 cm) olfactory cues to evaluate the presence or absence of an odor. While this dissertation focused on the olfactory tracking behaviors of fruit bats in the echolocating, neotropical leaf-nosed bats (Phyllostomidae), there is another large group of bats (Pteropodidae) who independently evolved frugivory and nectarivory (Teeling et al., 2005; Wang et al., 2020), while also losing the ability to echolocate (Thiagavel et al., 2018). While many pteropodid bats are demonstrated to use olfaction during food selection (Acharya et al., 1998; Luft et al., 2003; Tang et al., 2007), future work on their olfactory search mechanisms could provide further insight into how constraints imposed by echolocation and/or flight influence these behaviors.

Nocturnal, elusive, and mysterious, bats continue to fascinate and continue to offer an exciting opportunity to further understand mammalian sensory ecology. Learning the strategies bats use to navigate odors as fast flying organisms has implications for human development and technology (i.e., gas-seeking sniffer drones, Settles, 2005). Bats are also on global decline (Frick et al. 2019; Mickleburgh et al., 2002), in part due to habitat loss and fragmentation. Understanding how bats do or don't use sensory cues during critical tasks such as foraging are important for implementing conservation and management plans.

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APPENDIX A

SUPPLEMENTARY DATA FOR CHAPTER 2

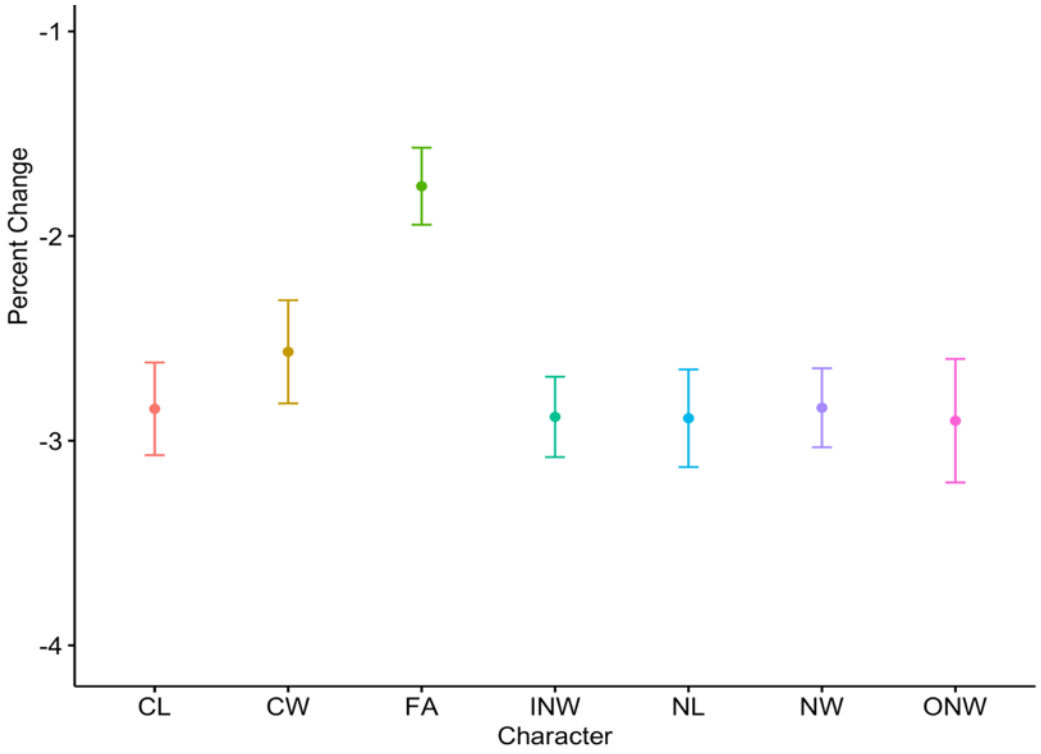

Figure A.1. Percent change between live and museum specimens for each morphological character. Percent change in forearm was significantly different compared to all other measurements except CW (post-hoc pairwise comparisons, $\alpha = 0.05$). CL, cranial length, CW: cranial width, FA: forearm, INW: inner nostril width, NL: nose length, NW: nose width, ONW: outer nostril width.

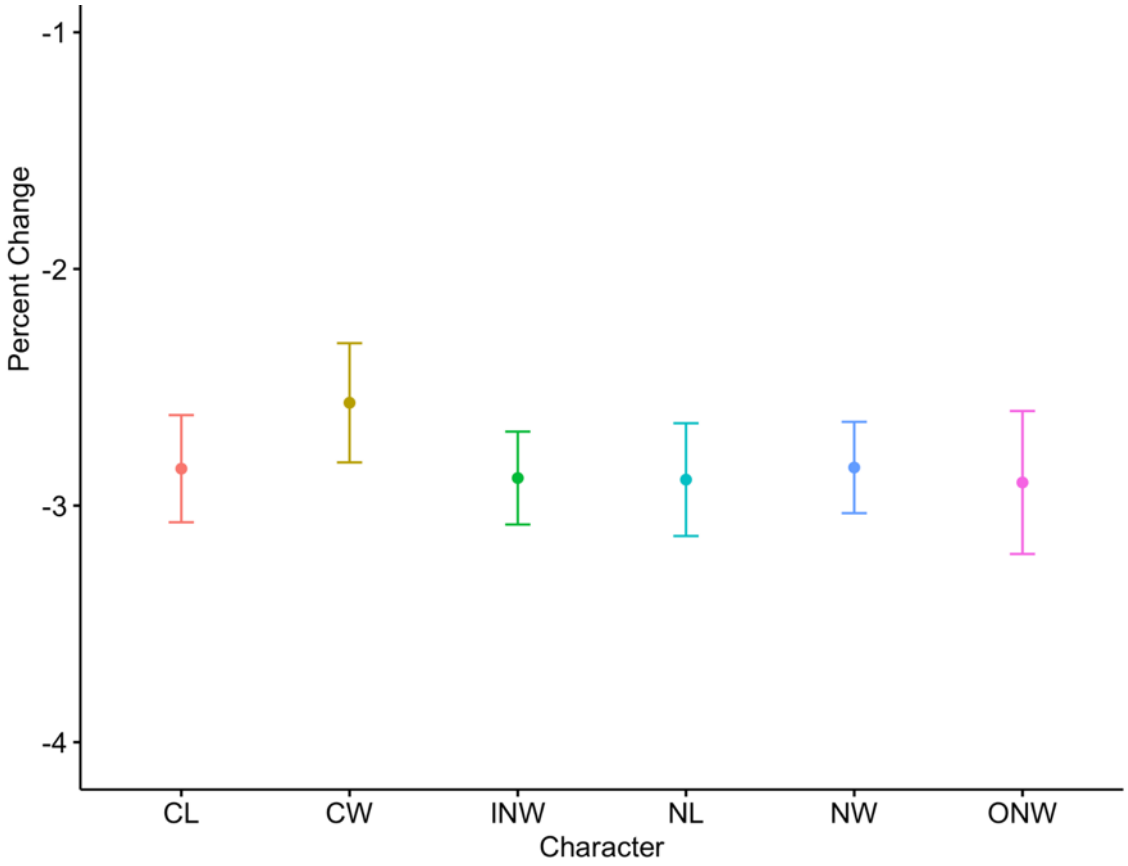

Figure A.2. Percent change between live and museum specimens for each morphological character. When forearm was excluded from the analysis, there was no significant difference in percent change across morphological measurements (one-way ANOVA, F = 0.289, *P* = 0.917). Abbreviations: CL, cranial length, CW: cranial width, INW: inner nostril width, NL: nose length, NW: nose width, ONW: outer nostril width.

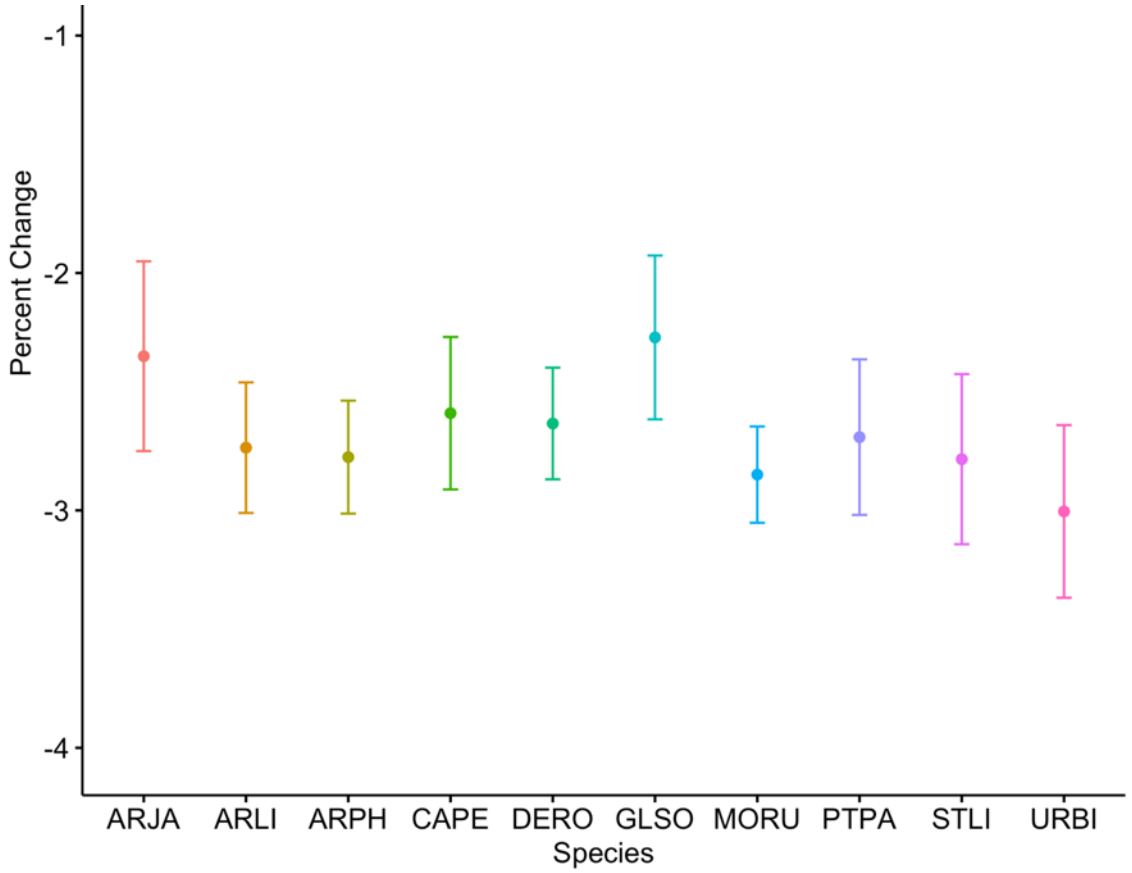

Figure A.3. Percent change between live and museum specimens for each species. There was no significant difference in percent change across different species (one-way ANOVA, F = 0.501, *P* = 0.868). Abbreviations: ARJA: *Artibeus jamaicensis*, ARLI: *A. lituratus,* ARPH: *A. phaeotis,* CAPE: *Carollia perspicillata,* DERO: *Desmodus rotundus*, GLSO: *Glossophaga soricina*, MORU: *Molossus rufus,* PTPA: *Pteronotus parnellii,* STLI: *Sturnira lilium*, URBI: *Uroderma bilobatum*.

Taxon	Sample Size	Body Mass (g)[1]	Diet Category	Forage Habitat	Forage Mode	Migrate ?	Echo Mode	Flight Speed (m/s)	Wing Load (g/cm^2)	Aspect Ratio
Eumops perotis	6	50.9	Insect[2]	Open	Aerial	No	Oral	6.57[3]	0.27[3]	9.98[3]
Molossus rufus	10	32[4]	Insect	Open	Aerial	No	Oral			
Nyctinomops macrotis	4	20.8	Insect[5]	Open	Aerial	No	Oral	8.94[5]		9.71[5]
Tadarida brasiliensis	10	12.2	Insect	Open	Aerial	Yes	Oral	5.19[3]	0.116[6]	8.6[6]
Mormoops megalophylla	10	16	Insect	Edge	Aerial	No	Oral	5.26[7]		7.5[8]
Pteronotus gymnonotus	10	13.6	Insect	Edge	Aerial	No	Oral			8.7[8]
Pteronotus parnellii	10	19.5	Insect[9]	Narrow	Aerial	No	Oral	4.87[7]		6.48[10]
Pteronotus personatus	10	5.6	Insect[11]	Edge	Aerial	No	Oral	4.31[7]		
Anoura geoffroyi	9	15	Nectar[12]	Narrow	Gleaning	No	Nasal			6.5[12]
Artibeus jamaicensis	10	41.6	Fruit[13]	Narrow	Gleaning	No	Nasal	3.75[14]	0.37[15]	6.4[8]
Artibeus lituratus	10	59.3	Fruit	Narrow	Gleaning	No	Nasal		0.39[15]	6.25[10]
Artibeus phaeotis	10	11.7	Fruit[16]	Narrow	Gleaning	No	Nasal		0.10[17]	6.33[17]
Carollia perspicillata	10	19.1	Fruit[18]	Narrow	Gleaning	No	Nasal	4.00[19]	0.25[15]	6.22[10]
Chiroderma trinitatum	5	23.6	Fruit	Narrow	Gleaning	No	Nasal			6.24[10]
Chiroderma villosum	3	25	Fruit[20]	Narrow	Gleaning	No	Nasal		0.14[17]	6.37[10]
Desmodus rotundus	10	33	Blood[21]	Edge	Gleaning	No	Nasal	3.75[22]	0.16[17]	6.73[23]
Diphylla ecaudata	9	28.11	Blood[24]	Edge	Gleaning	No	Nasal			
Glossophaga soricina	10	9.9	Nectar[25,26]	Narrow	Gleaning	No	Nasal	4.8[27]	0.09[17]	6.47[10]
Leptonycteris yerbabuenae	10	22.24	Nectar[28]	Narrow	Gleaning	Yes	Nasal	7.6[29]	0.16[30]	7.10[30]
Lonchophylla handleyi	9	17[31]	Nectar[32]	Narrow	Gleaning	No	Nasal			
Lonchorhina aurita	2	15.3	Insect[33]	Narrow	Gleaning	No	Nasal			
Macrotus waterhousii	10	16.1	Insect[34]	Narrow	Gleaning	No	Nasal			9.00[8]
Micronycteris megalotis	10	13.8	Insect[35]	Narrow	Gleaning	No	Nasal			5.74[10]
Mimon crenulatum	2	6.4	Insect	Narrow	Gleaning	No	Nasal			6.45[10]
Phyllostomus discolor	11	41.4	Omnivore[36]	Narrow	Gleaning	No	Nasal		0.15[36]	6.93[10]
Phyllostomus hastatus	10	91.1	Omnivore[37]	Narrow	Gleaning	No	Nasal	8.0[14]	0.20[38]	6.80[38]
Sturnira lilium	10	20.2	Fruit[39]	Narrow	Gleaning	No	Nasal	4.17[40]	0.13[17]	6.27[10]
Uroderma bilobatum	10	16.2	Fruit[41]	Narrow	Gleaning	No	Nasal		0.10[17]	6.3[10]
Vampyressa bidens	10	11.8	Fruit[42]	Narrow	Gleaning	No	Nasal		0.11[42]	6.38[10]
Vampyressa pusilla	2	8.6	Fruit[43]	Narrow	Gleaning	No	Nasal		0.11[43]	5.88[43]

Taxon	Sample Size	Body Mass (g)[1]	Diet Category	Forage Habitat	Forage Mode	Migrate ?	Echo Mode	Flight Speed (m/s)	Wing Load (g/cm^2)	Aspect Ratio
Antrozous pallidus	10	22.2	Omnivore[44]	Narrow	Gleaning	No	Oral	4.00[45]	0.12[6]	6.55[6]
Eptescicus fuscus	9	17.3	Insect[46]	Edge	Aerial	No	Oral	5.15[45]	0.09[6]	7.06[6]
Lasiurus borealis	10	12.3	Insect	Edge	Aerial	Yes	Oral	3.53[47]	0.08[6]	7.55[6]
Lasiurus cinereus	6	26.8	Insect[48]	Open	Aerial	Yes	Oral	5.05[45]	0.13[6]	8.25[6]
Lasiurus seminolus	7	9.87	Insect	Edge	Aerial	No	Oral			6.7[8]
Myotis nigricans	10	4.25[49]	Insect	Edge	Aerial	No	Oral	2.41[50]	0.07[8]	6.5[8]
Myotis velifer	11	9.78	Insect[51]	Edge	Aerial	No	Oral	4.51[45]	0.06[6]	6.73[6]
Myotis yumanensis	8	5.15	Insect	Edge	Aerial	No	Oral	3.88[45]	0.05[51]	6.45[45]
Pipstrellus subflavus	6	5.7	Insect	Edge	Aerial	No	Oral	4.35[47]	0.09[6]	6.92[6]
Rhogeessa tumida	8	4.58	Insect	Edge	Aerial	No	Oral		0.08[17]	6.2[17]

Table A.1. Variables used in this study, organized by species: sample size, body mass, diet category, foraging habitat, foraging mode, migration type, echolocation mode, flight speed, wing loading, and aspect ratio (where data was available).

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Table A.2 Phylogenetic signal for each of the various morphometric measurements, using the full dataset consisting of all 40 species. Italics indicate significance $P < 0.05$. Morphology character abbreviations are described in Table 2.1.

Character	Estimated Pagel's λ	*P*-value	Estimated Blomberg's K	*P*-value
Average Mass	0.045	0.813	0.509	0.313
INW	0.999	*<0.001*	1.594	*<0.001*
ONW	0.999	*0.002*	0.825	*<0.001*
NL	0.999	*0.018*	0.725	*0.016*
NW	0.872	*0.005*	0.769	*0.002*
CL	0.762	*<0.001*	0.845	*0.003*
CW	0.685	0.056	0.643	*0.046*
FA	0.384	0.356	0.567	0.073
INWR	0.999	*<0.001*	1.497	*<0.001*
NareW	0.399	*0.031*	0.666	*0.018*

Table A.3 Phylogenetic signal for each of the various morphometric measurements, from species within the family Phyllostomidae (n = 22). Italics indicate significance $P < 0.05$. Morphology character abbreviations are described in Table 2.1.

Character	Estimated Pagel's λ	*P*-value	Estimated Blomberg's K	*P*-value
Average Mass	~ 0	1	0.669	0.574
INW	0.999	0.129	0.950	0.061
ONW	0.481	1	0.798	0.238
NL	0.752	0.329	0.863	0.112
NW	0.516	0.820	0.799	0.204
CL	~ 0	1	0.621	0.629
CW	~ 0	1	0.573	0.735
FA	0.168	0.860	0.709	0.397
INWR	0.991	0.087	0.975	*0.039*
NareW	~ 0	1	0.664	0.559

Table A.4. Summary of morphological measurements for species included in this study. Morphology character abbreviations are described in Table 2.1. All measurements except INWR are in millimeters. INWR is a ratio and does not have specific units.

Taxon	INW	ONW	NL	NW	CL	CW	NareW	INWR	FA
Eumops perotis	5.59	9.05	19.04	10.54	23.61	25.32	1.73	4.53	76.35
Molossus rufus	2.47	4.11	7.55	5.68	16.52	16.36	0.82	6.74	49.73
Nyctinomops macrotis	4.39	5.85	8.21	7.10	16.04	14.01	0.73	3.20	61.85
Tadarida brasiliensis	2.92	4.14	7.91	5.29	13.04	10.12	0.61	3.47	42.91
Mormoops megalophylla	1.69	3.01	5.26	4.02	9.97	10.79	0.66	6.42	54.56
Pteronotus gymnonotus	1.22	2.79	6.80	4.09	11.72	10.00	0.79	8.30	52.79
Pteronotus parnellii	1.86	3.47	9.04	4.52	13.79	12.56	0.81	6.85	59.35
Pteronotus personatus	1.28	2.53	5.14	3.45	10.70	8.07	0.62	6.32	43.08
Anoura geoffroyi	0.77	2.10	8.29	4.51	15.75	11.04	0.67	14.66	42.46
Artibeus jamaicensis	1.79	4.49	7.78	7.49	19.51	15.17	1.35	8.48	59.59
Artibeus lituratus	1.85	4.86	7.18	7.83	19.39	15.90	1.51	8.71	64.65
Artibeus phaeotis	1.43	3.23	5.54	6.25	14.10	9.92	0.90	7.06	38.32
Carollia perspicillata	1.15	2.95	5.68	5.40	15.41	11.16	0.90	9.81	44.09
Chiroderma trinitatum	1.41	3.27	6.06	6.44	14.98	10.77	0.93	7.72	38.76
Chiroderma villosum	1.56	3.65	7.29	8.28	17.98	13.17	1.04	8.59	45.83
Desmodus rotundus	1.17	3.34	4.94	7.83	18.49	13.85	1.09	11.97	59.72
Diphylla ecaudata	1.35	3.53	3.07	5.68	19.08	12.86	1.09	9.56	54.83
Glossophaga soricina	1.08	2.75	5.66	4.21	14.27	10.56	0.83	9.91	35.92
Leptonycteris yerbabuenae	0.87	2.64	9.76	4.75	18.22	12.80	0.89	14.88	53.43
Lonchorhina aurita	1.04	3.09	7.39	4.86	15.43	11.76	1.02	11.45	45.75
Lonchophylla handleyi	1.19	3.23	10.82	4.77	17.18	12.05	1.02	10.15	44.92
Macrotus waterhousii	1.50	3.50	5.59	5.16	13.55	11.45	1.00	7.65	53.49
Mimon crenulatum	2.50	4.08	6.64	7.19	14.50	11.54	0.79	4.63	48.92
Micronycteris megalotis	0.94	2.36	6.11	5.02	11.29	9.15	0.71	9.80	35.65
Phyllostomus discolor	1.31	3.83	10.09	7.32	19.94	15.56	1.26	12.10	62.87
Phyllostomus hastatus	2.12	5.58	10.65	9.64	21.85	17.00	1.73	8.09	73.82
Sturnira lilium	1.29	3.32	4.95	4.81	15.23	12.29	1.02	9.53	38.92
Uroderma bilobatum	1.43	3.59	6.21	5.78	15.44	11.09	1.08	7.87	44.62
Vampyressa bidens	1.10	3.03	5.59	6.52	13.57	9.11	0.96	8.33	35.85
Vampyressa pusilla	0.91	2.15	4.39	4.65	11.75	8.92	0.62	9.81	31.63
Antrozous pallidus	1.97	3.29	5.96	5.58	13.67	12.49	0.66	6.38	50.78
Eptescius fuscus	2.30	3.88	7.44	4.66	12.54	11.62	0.79	5.11	48.89
Lasiurus borealis	2.11	3.32	4.15	4.19	9.38	9.52	0.61	4.53	40.12
Lasiurus cinereus	3.09	4.85	5.96	5.83	13.09	11.35	0.88	3.67	52.35
Lasiurus seminolus	2.14	3.34	3.88	4.14	10.16	9.55	0.60	4.47	40.00
Myotis nigricans	2.42	3.91	7.11	4.81	11.35	10.64	0.75	4.44	35.91
Myotis velifer	1.79	3.16	5.93	3.97	11.03	10.12	0.69	5.74	43.86
Myotis yumanensis	2.02	2.72	6.21	3.05	9.51	8.62	0.35	4.28	35.12

Taxon	INW	ONW	NL	NW	CL	CW	NareW	INWR	FA
Perimyotis subflavus	1.45	2.30	4.94	2.82	8.16	8.40	0.43	5.84	33.53
Rhogeessa tumida	1.65	2.88	5.58	3.58	9.49	8.87	0.62	5.45	30.70
Mean	1.80	3.58	6.89	5.54	14.52	11.87	0.89	7.66	47.65
SE	0.15	0.19	0.42	0.27	0.58	0.49	0.05	0.46	1.76
Rattus rattus	2.52	4.24	20.09	4.93	27.06	16.28	0.86	6.63	-
Mus musculus	1.50	2.39	9.65	3.10	16.28	10.63	0.44	7.43	-
Mean	2.01	3.31	14.87	4.01	21.67	13.46	0.65	7.03	-
SE	0.51	0.93	5.22	0.91	5.39	2.82	0.21	0.40	-

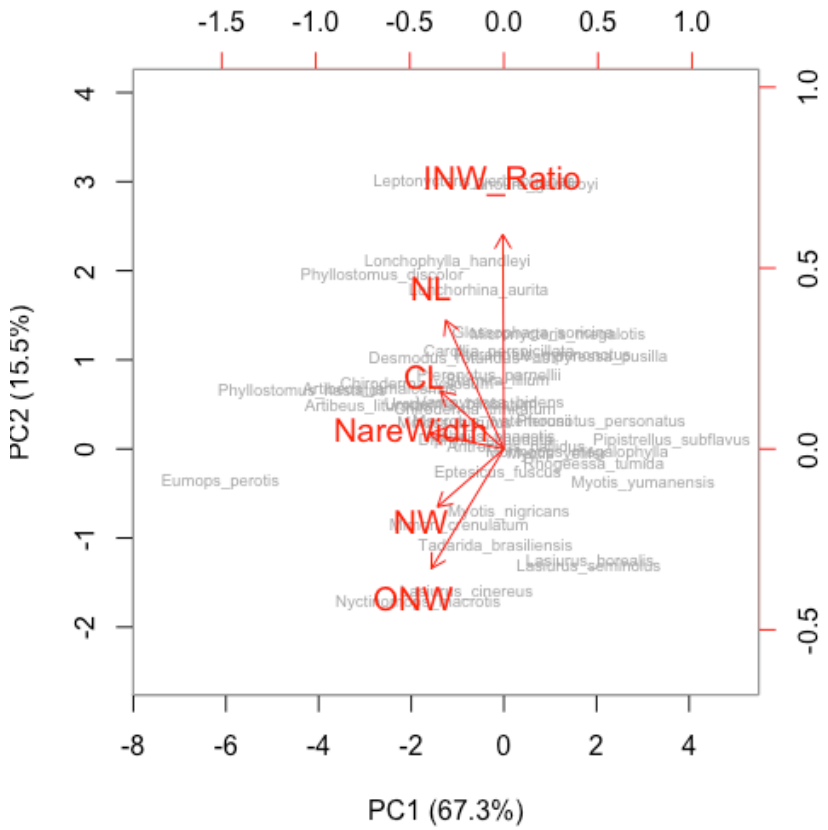

Figure A.4. Biplot of variable loadings from a phylogenetic principal component analysis on the full species dataset (n = 40). Morphology character abbreviations are described in Table 2.1.

Table A.5. Loadings and percent variance explained by each PC axis, obtained using a phylogenetic principal component analysis on the full dataset (n=40). Morphology character abbreviations are described in Table 2.1.

Character	PC1	PC2	PC3
ONW	-0.911	-0.375	0.050
NL	-0.708	0.390	0.587
NW	-0.897	-0.195	-0.123
CL	-0.906	0.205	-0.171
INWR	-0.018	0.906	0.412
NareW	-0.915	0.048	-0.282
Percent variation	67.3	15.5	10.9

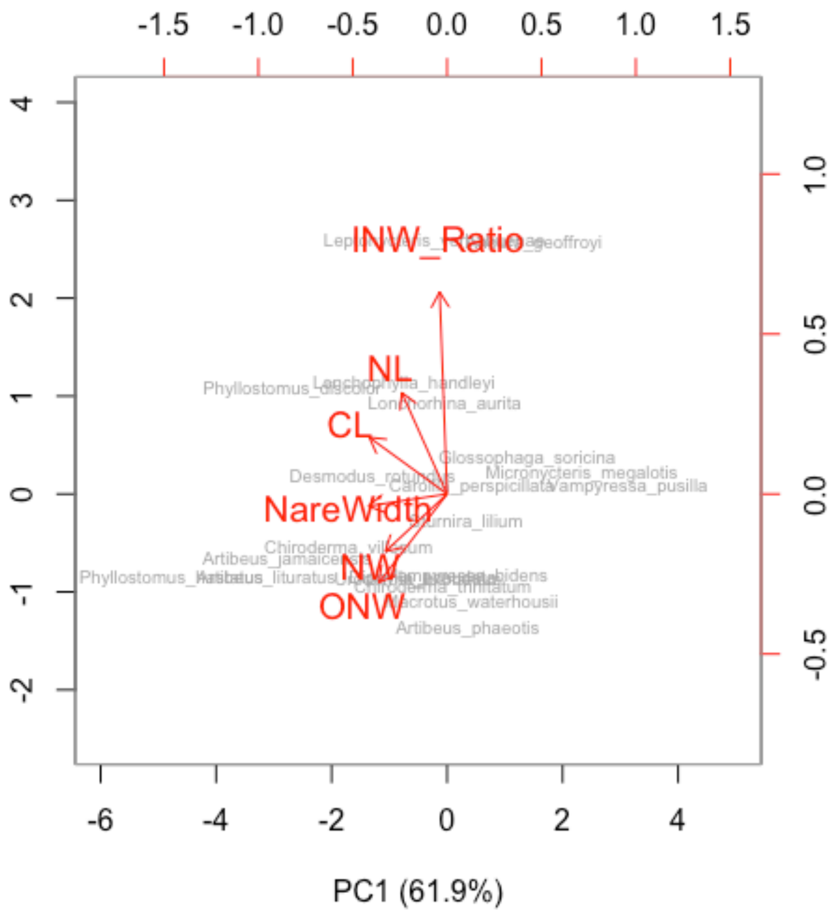

Figure A.5 Biplot of variable loadings from a phylogenetic principal component analysis on species in the family Phyllostomidae (n = 22). Morphology character abbreviations are described in Table 2.1.

Table A.6. Loadings and percent variance explained by each PC axis, obtained using a phylogenetic principal component analysis on species within Phyllostomidae (n=22). Morphology character abbreviations are described in Table 2.1

Character	PC1	PC2	PC3
ONW	-0.895	-0.409	-0.020
NL	-0.602	0.474	0.637
NW	-0.828	-0.271	0.074
CL	-0.922	0.236	-0.156
INWR	-0.099	0.947	-0.272
NareW	-0.939	-0.050	-0.187
Percent variation	61.9	22.1	8.6

Model: PC1	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Body Mass	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
BM + FH + M	120.34	0	26.08	$4.46e^{-10}$	0.720	$2.01e^{-11}$	-	0.033	-	-	0.0817
BM + FH	121.23	0.89	31.75	$3.24e^{-10}$	0.703	$3.26e^{-11}$	-	0.039	-	-	-
BM + FH + EM + M	122.00	1.66	21.04	$1.53e^{-9}$	0.719	$2.75e^{-11}$	-	0.033	-	0.330	0.083
BM	122.08	1.74	60.84	$2.09e^{-9}$	0.605	$2.08e^{-9}$	-	-	-	-	-
BM + FH + EM	122.82	2.48	23.99	$1.29e^{-9}$	0.702	$4.42e^{-11}$	-	0.039	-	0.344	-
BM + FH + FM + M	122.99	2.64	20.36	$2.33e^{-9}$	0.713	$3.74e^{-11}$	-	0.036	0.709	-	0.087
BM + FH + FM	123.70	3.36	23.27	$1.89e^{-9}$	0.695	$5.84e^{-11}$	-	0.042	0.718	-	-
BM + M	123.88	3.54	29.99	$1.81e^{-8}$	0.598	$3.29e^{-9}$	-	-	-	-	0.463
BM + FH + FM + EM + M	124.03	3.69	17.55	$5.26e^{-8}$	0.718	$4.06e^{-11}$	-	0.034	0.707	0.228	0.079
BM + EM	124.16	3.82	30.05	$1.77e^{-9}$	0.598	$3.03e^{-9}$	-	-	-	0.619	-
BM + FM	124.43	4.08	29.62	$2.09e^{-8}$	0.595	$3.41e^{-9}$	-	-	0.999	-	-
BM + FH + FM + EM	124.86	4.52	19.12	$5.05e^{-9}$	0.699	$6.69e^{-11}$	-	0.041	0.716	0.243	-
BM + FM + EM	126.14	5.79	20.02	$8.34e^{-8}$	0.594	$4.01e^{-9}$	-	-	0.982	0.402	-
BM + EM + M	126.14	5.79	19.72	$9.85e^{-8}$	0.590	$4.75e^{-9}$	-	-	-	0.635	0.496
BM + FM + M	126.35	6.01	19.45	$1.15e^{-7}$	0.587	$5.42e^{-9}$	-	-	0.983	-	0.463
BM + FM + EM + M	128.18	7.83	14.91	$3.32e^{-7}$	0.588	$5.77e^{-9}$	-	-	0.985	0.409	0.478
BM + D	130.95	10.61	11.51	$1.49e^{-6}$	0.574	$1.02e^{-8}$	0.881	-	-	-	-
BM + D + FH + M	132.46	12.12	11.59	$1.91e^{-7}$	0.685	$2.78e^{-10}$	0.872	0.072	-	-	0.112
BM + D + FH	132.47	12.13	12.23	$1.72e^{-7}$	0.668	$3.74e^{-10}$	0.882	0.080	-	-	-
BM + D + EM	132.71	12.36	9.995	$2.71e^{-6}$	0.581	$9.69e^{-9}$	0.879	-	-	0.301	-
BM + D + M	133.28	12.94	9.471	$4.62e^{-6}$	0.566	$1.57e^{-8}$	0.885	-	-	-	0.464
BM + D + FM	133.52	13.18	9.541	$4.29e^{-6}$	0.568	$1.39e^{-8}$	0.884	-	0.565	-	
BM + D + FH + EM	133.55	13.21	11.18	$2.84e^{-7}$	0.676	$3.79e^{-10}$	0.878	0.076	-	0.193	-

Model: PC1	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Body Mass	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
BM + D + FH + FM	135.33	14.99	10.52	$5.43e^{-7}$	0.661	$6.26e^{-10}$	0.886	0.085	0.563	-	-
BM + D + EM + M	135.34	14.99	8.486	$7.49e^{-6}$	0.573	$1.41e^{-8}$	0.883	-	-	0.303	0.524
BM + D + FH + FM + M	135.58	15.24	10.13	$5.82e^{-7}$	0.678	$4.76e^{-10}$	0.876	0.076	0.554	-	0.116
BM + D + FM + EM	135.64	15.30	8.45	$7.81e^{-6}$	0.572	$1.42e^{-8}$	0.885	-	0.521	0.354	-
BM + D + FM + M	136.12	15.78	8.05	$1.23e^{-5}$	0.559	$2.14e^{-8}$	0.887	-	0.582	-	0.509
BM + D + FH + FM + EM	136.78	16.44	9.73	$8.84e^{-7}$	0.668	$6.62e^{-10}$	0.882	0.081	0.559	0.209	-
BM + D + FH + FM + EM + M	137.07	16.73	9.52	$8.90e^{-7}$	0.686	$4.95e^{-10}$	0.871	0.072	0.549	0.197	0.112
BM + D + FM + EM + M	138.44	18.09	7.39	$1.82e^{-5}$	0.567	$1.90e^{-8}$	0.889	-	0.510	0.349	0.510
1	157.43	37.08	-	-	0	-	-	-	-	-	-

Table A.7. Summary of outputs from phylogenetic generalized least squares regression analysis on principal component (PC) 1 and ecological variables, with body mass (BM) as a size covariate. D: diet, FH: foraging habitat, FM: foraging mode, EM: echolocation mode, M: migratory type.

Model: PC1	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Forearm	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
FA + EM	110.41	0	48.68	$4.36e^{-11}$	0.709	$9.26e^{-12}$	-	-	-	0.1291	-
FA + FH	110.43	0.02	34.33	$1.17e^{-10}$	0.719	$7.89e^{-12}$	-	0.107	-	-	-
FA	110.59	0.18	91.55	$1.15e^{-11}$	0.699	$1.15e^{-11}$	-	-	-	-	-
FA + FH + EM	110.67	0.26	27.11	$2.71e^{-10}$	0.728	$7.10e^{-12}$	-	0.100	-	0.151	-
FA + FH + M	112.19	1.78	25.77	$5.22e^{-10}$	0.718	$1.17e^{-11}$	-	0.109	-	-	0.388
FA + FH + FM + EM	112.29	1.89	21.88	$9.47e^{-10}$	0.728	$9.83e^{-12}$	-	0.101	0.706	0.092	-
FA + FM + EM	112.35	1.94	32.16	$2.75e^{-10}$	0.706	$1.49e^{-11}$	-	-	0.461	0.138	-
FA + FM	112.35	1.95	45.48	$1.07e^{-10}$	0.695	$1.77e^{-11}$	-	-	0.468	-	-
FA + FH + EM + M	112.45	2.04	23.31	$4.24e^{-10}$	0.741	$1.36e^{-11}$	-	0.007	-	0.046	0.166
FA + EM + M	112.67	2.27	31.8	$3.18e^{-10}$	0.703	$1.66e^{-11}$	-	-	-	0.133	0.667
FA + M	112.71	2.30	44.92	$1.26e^{-10}$	0.693	$1.99e^{-11}$	-	-	-	-	0.653
FA + FH + FM	112.89	2.49	25.16	$7.07e^{-10}$	0.713	$1.47e^{-11}$	-	0.112	0.714	-	-
FA + FH + FM + EM + M	114.19	3.79	18.32	$3.11e^{-9}$	0.727	$1.43e^{-11}$	-	0.102	0.707	0.093	0.355
FA + FM + M	114.64	4.23	29.71	$7.16e^{-10}$	0.689	$3.13e^{-11}$	-	-	0.473	-	0.678
FA + FM + EM + M	114.73	4.32	23.65	$1.55e^{-9}$	0.699	$2.65e^{-11}$	-	-	0.466	0.142	0.645
FA + FH + FM + M	114.81	4.41	20.13	$2.69e^{-9}$	0.710	$2.19e^{-11}$	-	0.115	0.715	-	0.396
FA + D	117.47	7.06	18.4	$8.03e^{-9}$	0.691	$5.04e^{-11}$	0.571	-	-	-	-
FA + D + FH + EM	117.49	7.08	18.62	$7.60e^{-10}$	0.783	$1.22e^{-11}$	0.002	0.003	-	0.027	-
FA + D + EM	117.65	7.25	16.34	$1.23e^{-8}$	0.702	$4.19e^{-11}$	0.552	-	-	0.134	-
FA + D + FM + EM	118.90	8.49	14.47	$2.57e^{-8}$	0.707	$4.69e^{-11}$	0.545	-	0.701	0.059	-
FA + D + FH	119.82	9.42	14.03	$3.65e^{-8}$	0.701	$6.19e^{-11}$	0.556	0.224	-	-	-
FA + D + FM	120.25	9.85	14.97	$3.45e^{-8}$	0.683	$9.25e^{-11}$	0.583	-	0.712	-	-
FA + D + M	120.32	9.91	14.94	$3.54e^{-8}$	0.682	$9.43e^{-11}$	0.584	-	-	-	0.772
FA + D + EM + M	120.72	10.31	13.62	$5.14e^{-8}$	0.694	$8.11e^{-11}$	0.566	-	-	0.139	0.804

Model: PC1	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Forearm	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
FA + D + FH + FM + EM	120.85	10.45	16.12	$3.18e^{-9}$	0.777	$2.46e^{-11}$	0.003	0.004	0.185	0.068	-
FA + D + FH + FM	121.79	11.38	16.33	$3.76e^{-9}$	0.759	$4.44e^{-11}$	0.004	0.005	0.203	-	-
FA + D + FH + M	122.05	11.64	16.2	$4.14e^{-9}$	0.757	$4.79e^{-11}$	0.004	0.005	-	-	0.232
FA + D + FM + EM + M	122.16	11.75	12.3	$9.96e^{-8}$	0.699	$9.21e^{-11}$	0.559	-	0.705	0.064	0.786
FA + D + FH + FM + EM + M	122.54	12.13	14.97	$6.34e^{-9}$	0.782	$2.87e^{-11}$	0.003	0.003	0.182	0.066	0.215
FA + D + FM + M	123.30	12.89	12.48	$1.37e^{-7}$	0.673	$1.74e^{-10}$	0.596	-	0.716	-	0.784
FA + D + FH + FM + M	123.49	13.08	14.88	$8.14e^{-9}$	0.762	$5.32e^{-11}$	0.004	0.005	0.199	-	0.238
1	157.43	47.02	-	-	0	-	-	-	-	-	-

Table A.8. Summary of outputs from phylogenetic generalized least squares regression analysis on principal component (PC) 1 and ecological variables, with forearm (FA) as a size covariate. D: diet, FH: foraging habitat, FM: foraging mode, EM: echolocation mode, M: migratory type.

Model: PC2	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Body Mass	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
BM + D	85.01	0.00	5.50	0.001	0.366	0.063	0.001	-	-	-	-
BM + D + FH	86.32	1.32	4.74	0.001	0.402	0.056	0.001	0.148	-	-	-
BM + D + EM	86.36	1.36	4.85	0.001	0.372	0.062	0.001	-	-	0.255	-
BM + D + FM	87.61	2.60	4.53	0.002	0.352	0.066	0.001	-	0.592	-	-
BM + D + M	87.89	2.89	4.46	0.002	0.348	0.067	0.001	-	-	-	0.814
BM + D + FH + EM	88.69	3.69	4.22	0.002	0.398	0.058	0.001	0.150	-	0.386	-
BM + D + FM + EM	89.18	4.17	4.10	0.003	0.358	0.062	0.001	-	0.590	0.265	-
BM + D + EM + M	89.46	4.45	4.04	0.003	0.353	0.066	0.001	-	-	0.262	0.840
BM + D + FH + M	89.66	4.65	4.02	0.002	0.383	0.060	0.001	0.157	-	-	0.894
BM + D + FH + FM	89.68	4.67	4.02	0.002	0.383	0.061	0.001	0.157	0.960	-	-
BM + D + FM + M	90.69	5.69	3.78	0.004	0.333	0.070	0.001	-	0.597	-	0.828
BM + D + FH + FM + EM	91.66	6.65	3.74	0.003	0.387	0.060	0.001	0.155	0.959	0.277	-
BM + D + FM + EM + M	92.48	7.47	3.49	0.006	0.338	0.069	0.001	-	0.596	0.273	0.839
BM + D + FH + FM + M	93.24	8.23	3.46	0.005	0.362	0.065	0.001	0.166	0.960	-	0.897
BM + D + FH + FM + EM + M	95.47	10.47	3.26	0.006	0.366	0.064	0.001	0.165	0.960	0.285	0.889
BM + FH	95.52	10.52	2.57	0.069	0.108	0.114	-	0.092	-	-	-
BM	96.00	10.99	2.43	0.128	0.035	0.128	-	-	-	-	-
1	96.26	11.25	-	-	0.000	-	-	-	-	-	-
BM + EM	96.49	11.48	2.12	0.135	0.054	0.124	-	-	-	0.194	-
BM + FM	97.08	12.07	1.81	0.177	0.040	0.127	-	-	0.283	-	-
BM + FH + M	97.25	12.24	2.12	0.103	0.099	0.115	-	0.094	-	-	0.379
BM + FH + EM	97.71	12.71	1.99	0.117	0.092	0.117	-	0.096	-	0.541	-
BM + M	98.10	13.09	1.30	0.285	0.015	0.132	-	-	-	-	0.640
BM + FH + FM	98.15	13.14	1.88	0.137	0.082	0.119	-	0.099	0.995	-	-

Model: PC2	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Body Mass	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
BM + EM + M	98.67	13.66	1.47	0.239	0.035	0.128	-	-	-	0.198	0.608
BM + FM + EM	98.95	13.94	1.38	0.265	0.028	0.129	-	-	0.286	0.462	-
BM + FM + M	99.22	14.22	1.29	0.294	0.022	0.131	-	-	0.288	-	0.587
BM + FH + EM + M	99.59	14.59	1.74	0.153	0.086	0.119	-	0.098	-	0.542	0.386
BM + FH + FM + EM	100.01	15.00	1.65	0.174	0.077	0.120	-	0.101	0.995	0.381	-
BM + FH + FM + M	100.03	15.02	1.65	0.175	0.076	0.121	-	0.101	0.995	-	0.386
BM + FM + EM + M	101.26	16.25	1.08	0.381	0.008	0.133	-	-	0.292	0.467	0.606
BM + FH + FM + EM + M	102.11	17.11	1.48	0.215	0.069	0.122	-	0.103	0.995	0.383	0.406

Table A.9. Summary of outputs from phylogenetic generalized least squares regression analysis on principal component (PC) 2 and ecological variables, with body mass (BM) as a size covariate. D: diet, FH: foraging habitat, FM: foraging mode, EM: echolocation mode, M: migratory type.

Model: PC2	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Forearm	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
FA + D	86.30	0.00	5.10	0.001	0.345	0.470	0.001	-	-	-	
FA + D + EM	87.81	1.51	4.48	0.002	0.349	0.469	0.001	-	-	0.278	-
FA + D + FM	88.99	2.68	4.19	0.003	0.329	0.475	0.001	-	0.638	-	-
FA + D + FH	89.26	2.95	4.09	0.003	0.356	0.466	0.001	0.284	-	-	-
FA + D + M	89.26	2.95	4.13	0.003	0.325	0.477	0.001	-	-	-	0.965
FA + D + FM + EM	90.57	4.27	3.81	0.004	0.335	0.473	0.001	-	0.637	0.268	-
FA + D + EM + M	90.96	4.65	3.72	0.005	0.328	0.476	0.001	-	-	0.286	0.993
FA + D + FH + EM	91.80	5.50	3.61	0.004	0.349	0.469	0.001	0.288	-	0.432	-
FA + D + FM + M	92.13	5.83	3.49	0.007	0.308	0.482	0.001	-	0.643	-	0.979
FA + D + FH + M	92.49	6.18	3.49	0.006	0.338	0.473	0.001	0.294	-	-	0.758
FA + D + FH + FM	92.59	6.29	3.47	0.006	0.336	0.473	0.001	0.295	0.901	-	-
FA + D + FM + EM + M	93.93	7.62	3.23	0.009	0.313	0.481	0.001	-	0.642	0.275	0.986
FA + D + FH + FM + EM	94.46	8.15	3.26	0.007	0.343	0.471	0.001	0.292	0.900	0.260	-
FA + D + FH + FM + M	96.05	9.75	3.00	0.011	0.316	0.479	0.001	0.306	0.902	-	0.762
1	96.26	9.95	-	-	0.000	-	-	-	-	-	-
FA	98.11	11.81	0.34	0.561	-0.017	0.561	-	-	-	-	-
FA + D + FH + FM + EM + M	98.16	11.86	2.86	0.013	0.322	0.478	0.001	0.303	0.902	0.268	0.756
FA + FH	98.24	11.93	1.62	0.203	0.045	0.549	-	0.121	-	-	-
FA + EM	98.28	11.98	1.21	0.310	0.011	0.556	-	-	-	0.159	-
FA + FM	98.70	12.39	1.01	0.375	0.000	0.558	-	-	0.205	-	-
FA + FH + M	99.69	13.38	1.47	0.231	0.046	0.549	-	0.121	-	-	0.315
FA + M	100.02	13.71	0.37	0.692	-0.033	0.564	-	-	-	-	0.528
FA + FH + EM	100.22	13.92	1.34	0.275	0.034	0.551	-	0.124	-	0.458	-
FA + EM + M	100.26	13.96	0.94	0.429	-0.004	0.559	-	-	-	0.163	0.506

Model: PC2	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-values					
						Forearm	Diet	Forage Habitat	Forage Mode	Echo Mode	Migrate Type
FA + FM + M	100.63	14.33	0.83	0.488	-0.014	0.560	-	-	0.209	-	0.487
FA + FM + EM	100.67	14.36	0.81	0.494	-0.014	0.561	-	-	0.209	0.502	-
FA + FH + FM	100.80	14.49	1.20	0.330	0.020	0.554	-	0.128	0.816	-	-
FA + FH + EM + M	101.84	15.53	1.27	0.299	0.034	0.551	-	0.125	-	0.458	0.324
FA + FH + FM + M	102.40	16.10	1.16	0.349	0.020	0.554	-	0.128	0.816	-	0.321
FA + FM + EM + M	102.76	16.46	0.72	0.586	-0.030	0.564	-	-	0.212	0.505	0.502
FA + FH + FM + EM	102.77	16.46	1.09	0.386	0.011	0.556	-	0.130	0.817	0.411	-
FA + FH + FM + EM + M	104.58	18.28	1.06	0.404	0.010	0.556	-	0.131	0.817	0.411	0.335

Table A.10. Summary of outputs from phylogenetic generalized least squares regression analysis on principal component (PC) 2 and ecological variables, with forearm (FA) as a size covariate. D: diet, FH: foraging habitat, FM: foraging mode, EM: echolocation mode, M: migratory type.

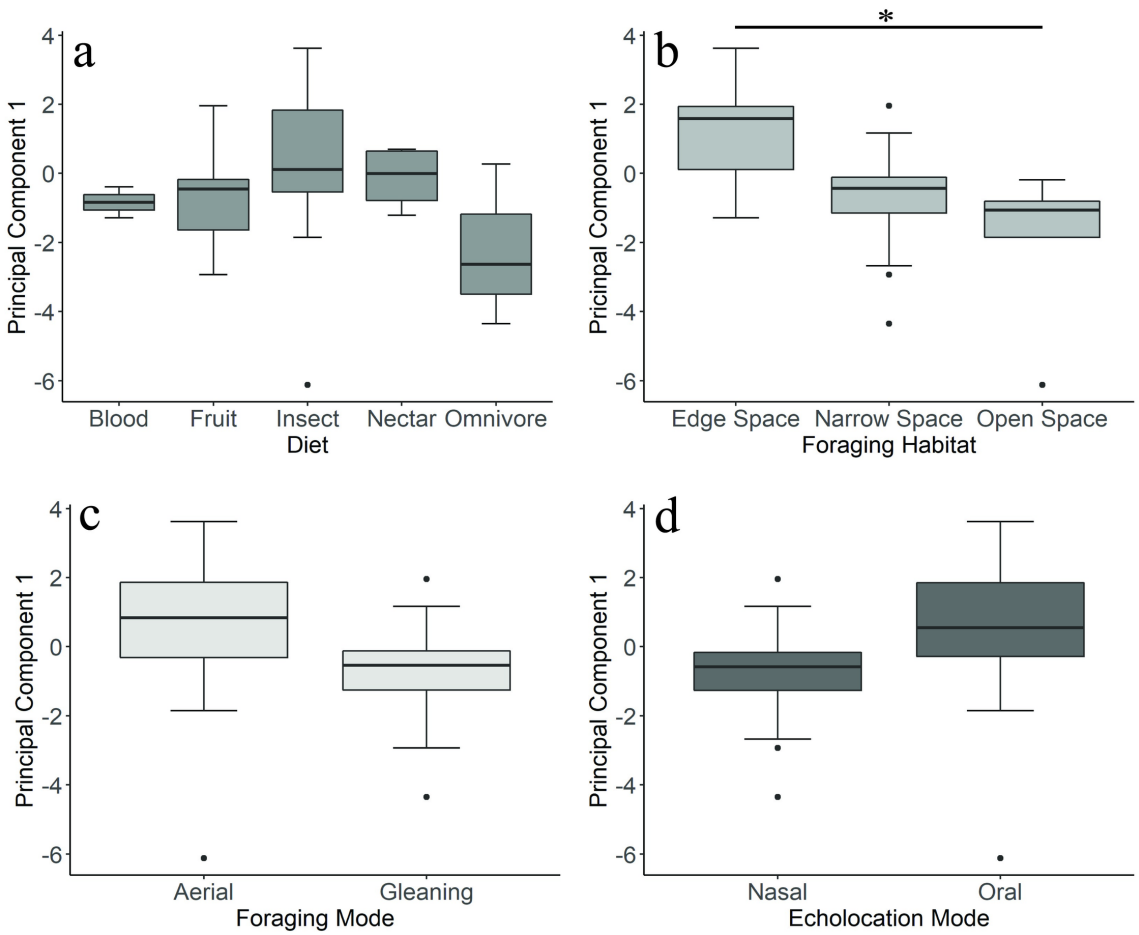

Figure A.6. Boxplots representing variation in PC1 from the full dataset: a) Diet, b) foraging habitat, c) foraging mode, d) echolocation mode.

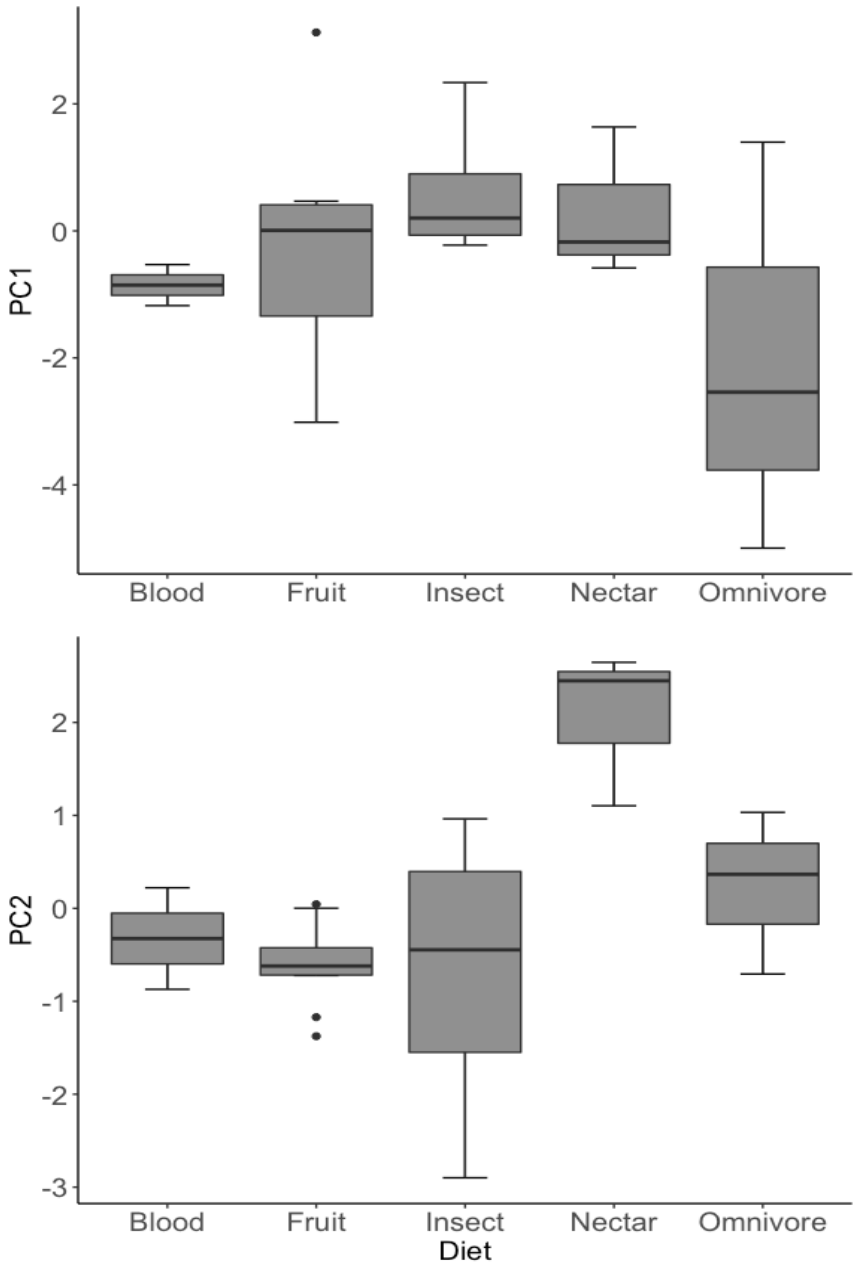

Figure A.7. Boxplots representing variation in phylogenetic Principal Component (PC) 1 (top) and PC 2 (bottom) for species within the species Phyllostomidae across diet categories.

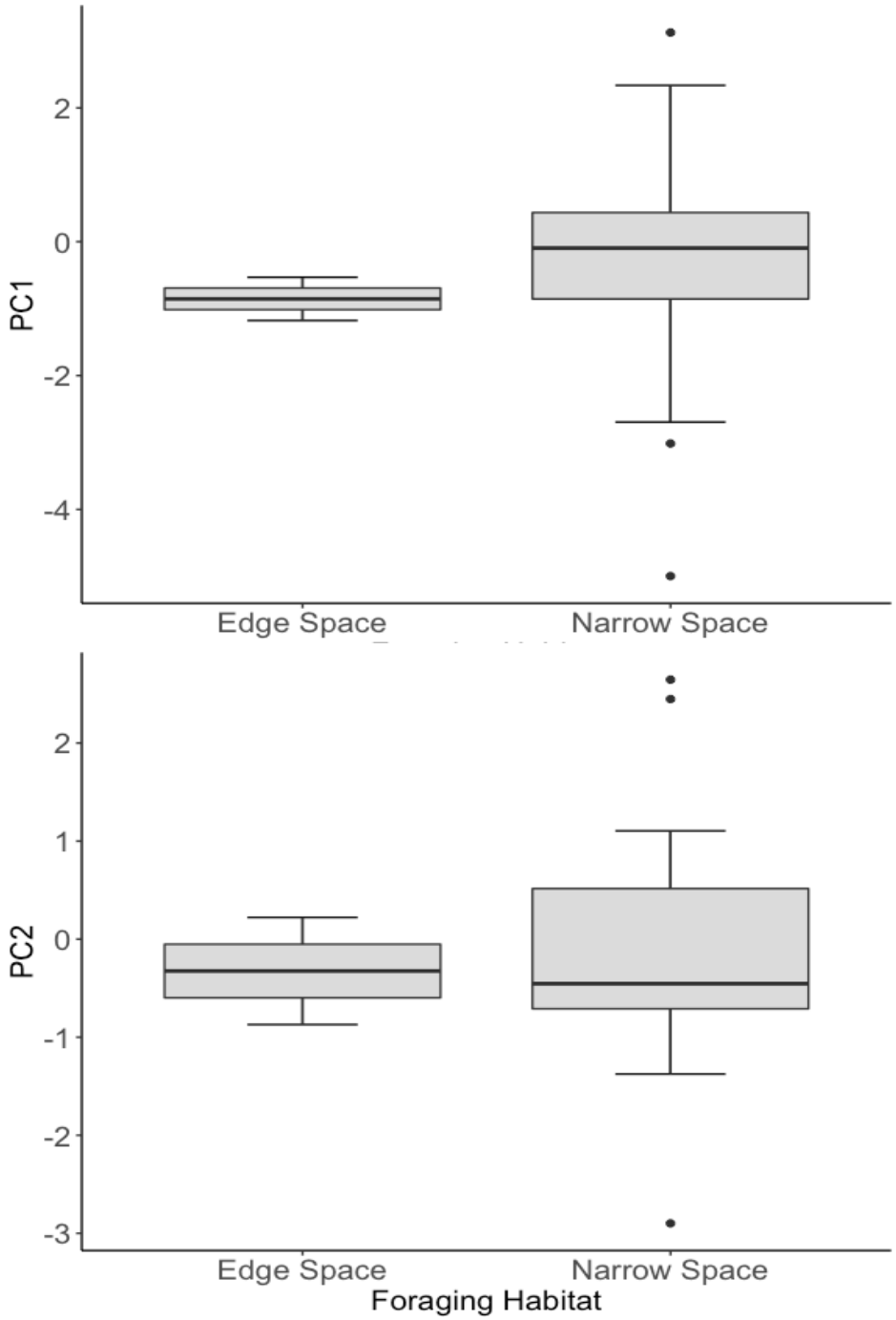

Figure A.8. Boxplots representing variation in phylogenetic Principal Component (PC) 1 (top) and PC 2 (bottom) for species within the species Phyllostomidae across foraging habitat categories.

Models	AICc	ΔAICc	F-stat	Adjusted R^2	Model P-value	P-values			
						Body Mass	Diet	Forage Habitat	Migrate Type
PC1 ~ BM	63.44	0.00	59.59	0.736	$2.01e^{-7}$	$2.01e^{-7}$	-	-	-
PC1 ~ BM + FH	65.92	2.48	28.54	0.724	$1.89e^{-6}$	$3.98e^{-7}$	-	0.657	-
PC1 ~ BM + M	66.05	2.61	28.44	0.723	$1.94e^{-6}$	$4.03e^{-7}$	-	-	0.775
PC1 ~ BM + FH + M	68.80	5.36	18.12	0.709	$1.13e^{-5}$	$8.01e^{-7}$	-	0.651	0.735
PC1 ~ BM + D	74.97	11.53	10.32	0.689	0.0001	$2.49e^{-6}$	0.889	-	-
PC1 ~ BM + D + M	79.37	15.93	8.06	0.669	0.001	$5.24e^{-6}$	0.899	-	0.991
PC1 ~ 1	90.35	26.91	-	0	-	-	-	-	-
PC2 ~ BM + M	68.70	0.00	3.00	0.159	0.074	0.183	-	-	0.057
PC2 ~ BM + D	68.98	0.29	3.43	0.366	0.027	0.131	0.027	-	-
PC2 ~ 1	69.60	0.90		0	-	-	-	-	-
PC2 ~ BM	70.28	1.59	1.65	0.030	0.213	0.233	-	-	-
PC2 ~ BM + FH + M	71.31	2.61	2.04	0.129	0.144	0.191	-	0.466	0.069
PC2 ~ BM + FH	72.43	3.73	1.05	0.005	0.369	0.219	-	0.494	-
PC2 ~ BM + D + M	72.83	4.13	2.81	0.341	0.049	0.139	0.033	-	0.546

Table A.11. Summary of outputs from phylogenetic generalized least squares regression analysis on principal components and ecological variables for species within the family Phyllostomidae (n = 22 species), using body mass (BM) as a covariate. D: diet, FH: foraging habitat, M: migratory type.

Models	AICc	ΔAICc	F-stat	Adjusted R^2	Model P-value	P-values			
						Forearm	Diet	Forage Habitat	Migrate Type
PC1 ~ FA	55.04	0.00	100.20	0.825	$3.12e^{-9}$	$3.12e^{-9}$	-	-	-
PC1 ~ FA + M	55.54	0.50	53.60	0.833	$1.54e^{-8}$	$3.49e^{-9}$	-	-	0.173
PC1 ~ FA + FH	56.75	1.71	50.22	0.824	$2.60e^{-8}$	$5.46e^{-9}$	-	0.361	-
PC1 ~ FA + FH + M	57.01	1.97	36.75	0.836	$6.96e^{-8}$	$5.33e^{-9}$	-	0.344	0.138
PC1 ~ FA + D	58.73	3.69	26.12	0.857	$3.66e^{-7}$	$6.69e^{-9}$	0.1284	-	-
PC1 ~ 1	90.35	35.31	-	0	-	-	-	-	-
PC2 ~ FA + D	69.18	0.00	4.53	0.457	0.009	0.888	0.005	-	-
PC2 ~ 1	69.60	0.42	-	0	-	-	-	-	-
PC2 ~ FA + M	70.00	0.82	2.28	0.108	0.129	0.486	-	-	0.059
PC2 ~ FA	71.55	2.37	0.44	-0.027	0.515	0.515	-	-	-
PC2 ~ FA + D + M	72.36	3.18	3.88	0.452	0.015	0.885	0.006	-	0.371
PC2 ~ FA + FH + M	72.80	3.62	1.51	0.068	0.245	0.496	-	0.522	0.073
PC2 ~ FA + FH	73.82	4.65	0.40	-0.061	0.676	0.522	-	0.548	-

Table A.12. Summary of outputs from phylogenetic generalized least squares regression analysis on principal components and ecological variables for species within the family Phyllostomidae (n = 22 species), using forearm (FA) as a covariate. D: diet, FH: foraging habitat, M: migratory type.

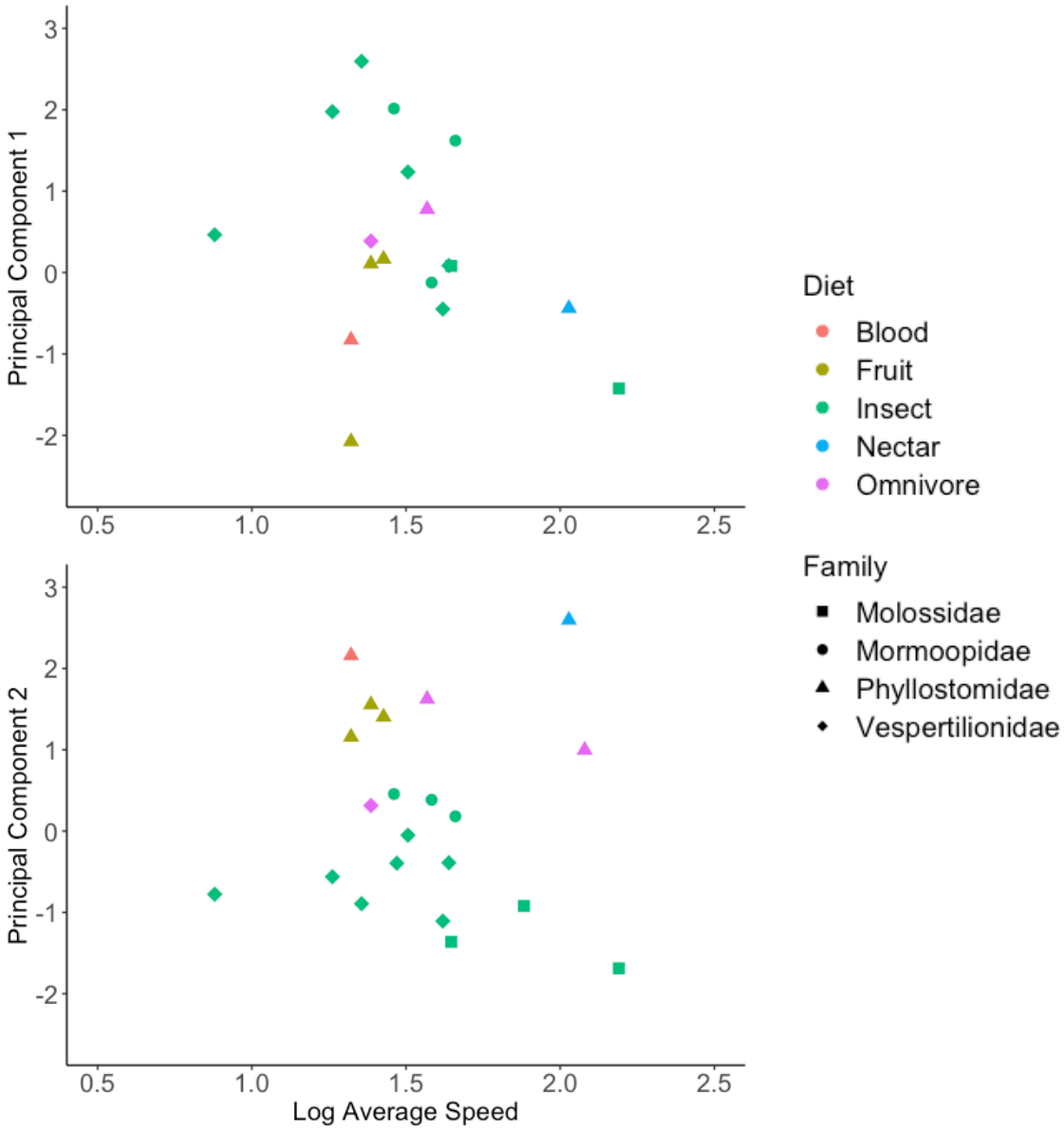

Figure A.10. Phylogenetic principal component (PC) 1 (top) and PC2 (bottom) plotted against log average speed for a subset of dataset (n = 21 species).

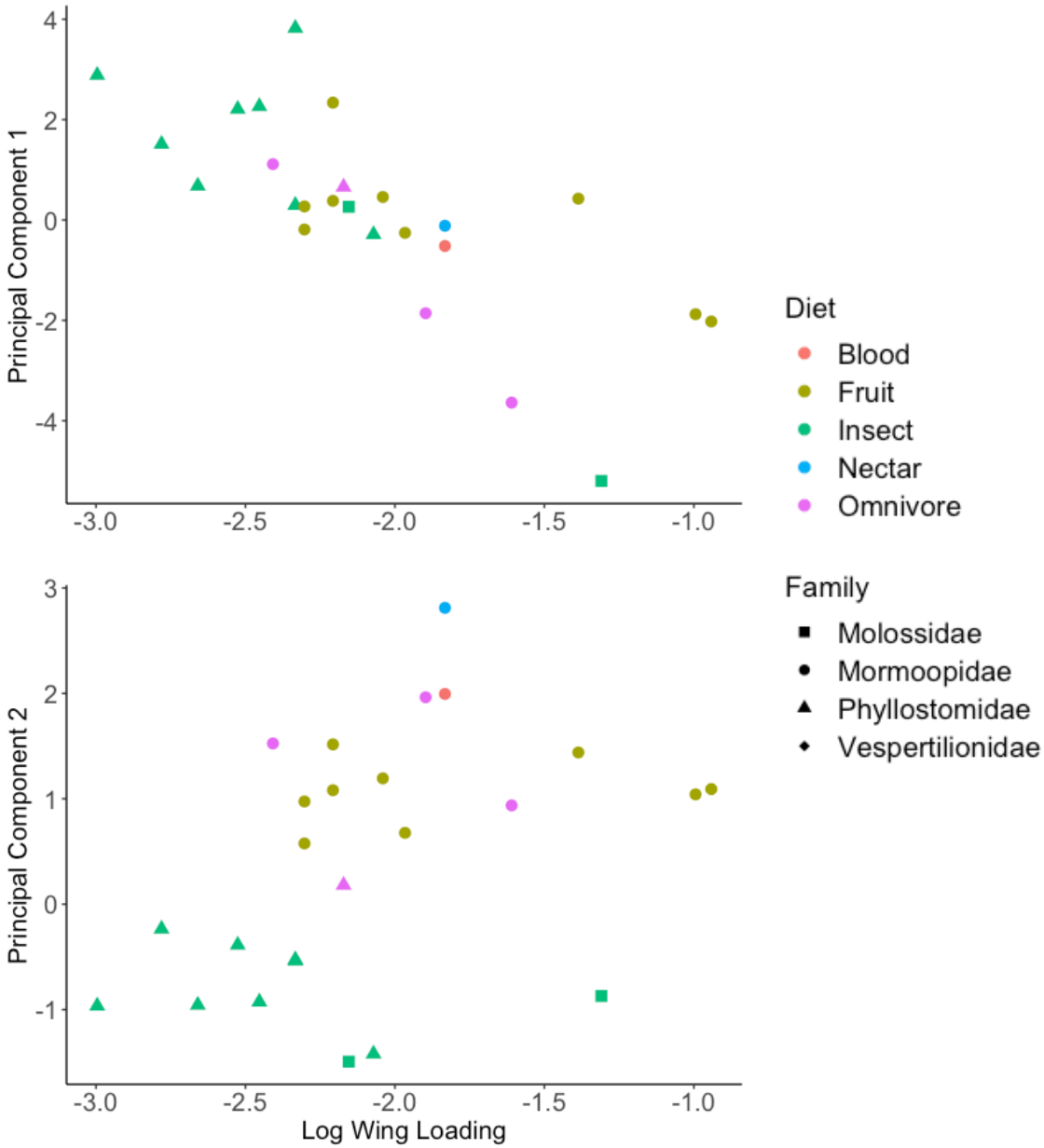

Figure A.11. Phylogenetic principal component (PC) 1 (top) and PC2 (bottom) plotted against log wing loading for a subset of dataset (n = 25 species).

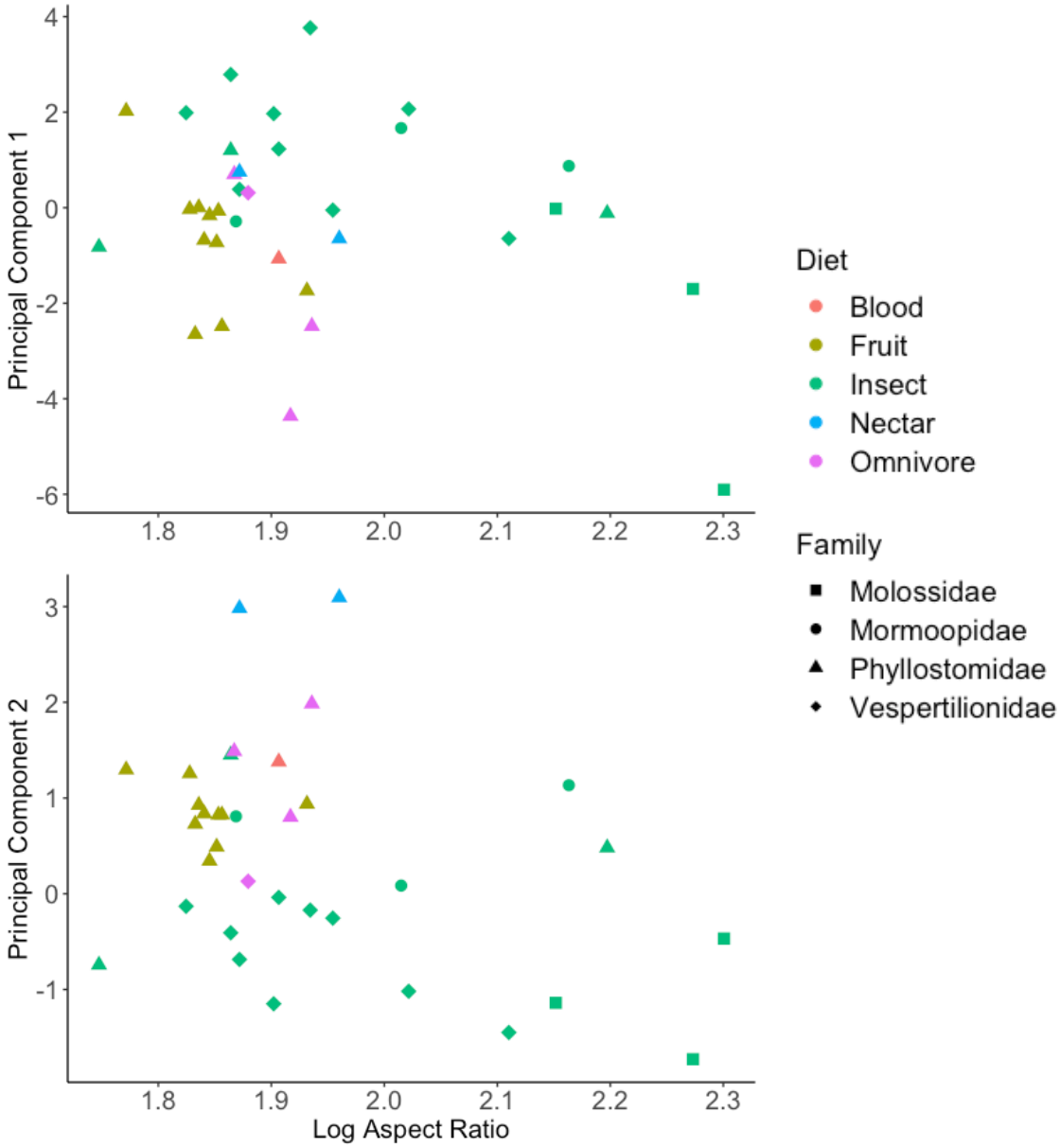

Figure A.12. Phylogenetic principal component (PC) 1 (top) and PC2 (bottom) plotted against log aspect ratio for a subset of dataset (n = 35 species

Flight Speed	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-value		
						Body Mass	Log Speed	Diet
PC1 ~ 1	88.36	23.21	-	-	0	-	-	-
PC1 ~ BM	65.15	0	45.60	$1.88e^{-6}$	0.690	$1.88e^{-6}$	-	-
PC1 ~ BM + S	67.71	2.56	21.85	$1.53e^{-5}$	0.676	$3.45e^{-6}$	0.593	-
PC1 ~ BM + S + D	79.62	14.47	7.321	0.001	0.655	$1.94e^{-5}$	0.253	0.614
PC2 ~ BM + S + D	48.89	0	4.69	0.008	0.525	0.342	0.428	0.003
PC2 ~ 1	49.71	0.82	-	-	0	-	-	-
PC2 ~ BM	51.32	2.43	0.82	0.376	-0.009	0.376	-	-
PC2 ~ BM + S	53.96	5.07	0.44	0.656	-0.059	0.388	0.771	-
						Forearm	Log Speed	Diet
PC1 ~ FA	65.92	0	43.22	$2.71e^{-6}$	0.679	$2.71e^{-6}$	-	-
PC1 ~ FA + S	66.82	0.90	24.21	$7.89e^{-6}$	0.699	$2.33e^{-6}$	0.145	-
PC1 ~ FA + S + D	77.84	11.92	8.175	0.001	0.683	$1.16e^{-5}$	0.160	0.561
PC2 ~ FA + S + D	48.95	0	4.62	0.009	0.521	0.686	0.617	0.003
PC2 ~ FA	51.71	2.76	0.46	0.508	-0.028	0.508	-	-
PC2 ~ FA + S	54.42	5.47	0.23	0.795	-0.083	0.519	0.859	-

Table A.13. Summary of outputs from phylogenetic generalized least squares regression analysis on principal components and log flight speed (n = 21 species).

Wing Loading	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-value		
						Body Mass	Wing Loading	Diet
PC1 ~ BM	74.75	0	56.94	$1.15e^{-7}$	0.699	$1.15e^{-7}$		
PC1 ~ BM + WL	76.99	2.24	27.82	$9.47e^{-7}$	0.691	$1.94e^{-7}$	0.582	
PC1 ~ BM + WL + D	88.08	13.33	8.681	0.0001	0.658	$1.35e^{-6}$	0.601	0.774
PC1 ~ 1	102.27	27.52			0			
PC2 ~ BM + WL + D	44.78	0	23.37	$1.42e^{-7}$	0.850	0.0001	0.041	$1.55e^{-7}$
PC2 ~ 1	53.95	9.16			0			
PC2 ~ BM	55.27	10.48	0.99	0.331	-0.001	0.331		
PC2 ~ BM + WL	57.65	12.86	0.57	0.572	-0.037	0.339	0.664	
						Forearm	Wing Loading	Diet
PC1 ~ FA	69.75	0	72.9	$1.38e^{-8}$	0.750	$1.38e^{-8}$		
PC1 ~ FA + WL	72.01	2.26	36.29	$1.08e^{-7}$	0.750	$2.14e^{-8}$	0.546	
PC1 ~ FA + WL + D	81.39	11.64	12.2	$1.69e^{-5}$	0.740	$1.33e^{-7}$	0.546	0.545
PC2 ~ FA + WL + D	45.41	0	22.72	$1.76e^{-7}$	0.845	0.022	0.0001	$2.19e^{-7}$
PC2 ~ FA	54.74	9.34	1.49	0.234	0.020	0.234		
PC2 ~ FA + WL	57.29	11.89	0.74	0.489	-0.022	0.244	0.841	

Table A.14. Summary of outputs from phylogenetic generalized least squares regression analysis on principal components and log wing loading (n = 25 species).

Aspect Ratio	AICc	ΔAICc	F-stat	Model P-value	Adjusted R^2	P-value		
						Body Mass	Aspect Ratio	Diet
PC1 ~ BM	107.79	0	56.00	$1.33e^{-8}$	0.618	$1.33e^{-8}$	-	-
PC1 ~ BM + AR	109.85	2.05	27.66	$1.06e^{-7}$	0.611	$1.94e^{-8}$	0.574	-
PC1 ~ BM + AR + D	119.94	12.14	8.52	$2.60e^{-5}$	0.570	$1.12e^{-7}$	0.596	0.899
PC1 ~ 1	139.59	31.80	-	-	0	-	-	-
PC2 ~ BM + AR + D	71.29	0	5.54	0.001	0.445	0.036	0.707	0.0004
PC2 ~ BM	82.02	10.73	2.85	0.101	0.051	0.101	-	-
PC2 ~ 1	82.66	11.37	-	-	0	-	-	-
PC2 ~ BM + AR	84.33	13.04	1.42	0.255	0.024	0.106	0.777	-
						Forearm	Aspect Ratio	Diet
PC1 ~ FA	98.68	0	80.27	$2.36e^{-10}$	0.699			
PC1 ~ FA + AR	101.08	2.39	38.92	$2.69e^{-9}$	0.691	$4.42e^{-10}$	0.955	-
PC1 ~ FA + AR + D	108.68	9.99	13.18	$4.81e^{-7}$	0.682	$1.85e^{-9}$	0.955	0.537
PC2 ~ FA + AR + D	71.28	0	5.55	0.001	0.445	0.268	0.942	0.0001
PC2 ~ FA	84.18	12.90	0.70	0.408	-0.009	0.408	-	-
PC2 ~ FA + AR	86.58	15.30	0.34	0.712	-0.040	0.415	0.958	-

Table A.15. Summary of outputs from phylogenetic generalized least squares regression analysis on principal components and log aspect ratio (n = 35 species).