Received: 5 February 2023 | Revised: 23 June 2023 | Accepted: 5 July 2023

DOI: 10.1002/ar.25290

SPECIAL ISSUE ARTICLE

The Anatomical Record WILEY

An updated synthesis of and outstanding questions in the olfactory and vomeronasal systems in bats: Genetics asks questions only anatomy can answer

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Funding information
Division of Integrative Organismal Systems, Grant/Award Number: 2032073

Abstract

The extensive diversity observed in bat nasal chemosensory systems has been well-documented at the histological level. Understanding how this diversity evolved and developing hypotheses as to why particular patterns exist require a phylogenetic perspective, which was first outlined in the work of anatomist Kunwar Bhatnagar. With the onset of genetics and genomics, it might be assumed that the puzzling patterns observed in the morphological data have been clarified. However, there is still a widespread mismatch of genetic and morphological correlations among bat chemosensory systems. Novel genomic evidence has set up new avenues to explore that demand more evidence from anatomical structures. Here, we outline the progress that has been made in both morphological and molecular studies on the olfactory and vomeronasal systems in bats since the work of Bhatnagar. Genomic data of olfactory and vomeronasal receptors demonstrate the strong need for further morphological sampling, with a particular focus on receiving brain regions, glands, and ducts.

KEYWORDS

bats, chemoreceptor, chemosensation, olfactory receptor, vomeronasal organ

1 | INTRODUCTION

Bats are well known for their exceptional sensory adaptations (Jones et al., 2013; Page & Hofstede, 2021); their ability to echolocate sets them apart from many other mammalian clades. Of the more than 1300 bat species (Burgin et al., 2018), most are insectivorous and primarily rely on ultrasonic signaling and perception to find their food, among other essential behaviors to fitness (Jones et al., 2013). However, the two independent origins of plant-visiting in bats, once in the Old World Pteropodidae clade and a second time within the neotropical Phyllostomidae (Nesi et al., 2021; Rojas et al., 2016; Shi & Rabosky, 2015), brings another sensory system into the light: chemosensation. Ultrasonic signaling is useful for finding moving targets in the air, but perhaps less so for identifying a ripened, stagnant fruit hidden under a leaf in a cluttered forest. Behaviorally, both clades of bats have been described to primarily use the sense of smell to locate food targets, though these are often species-specific studies (Gonzalez-Terrazas et al., 2016; Hodgkison et al., 2013; Korine &

Kalko, 2005). Thus, given a shift from a presumed insectivorous ancestor relying primarily on auditory cues (Dumont et al., 2012), it is expected that the morphological and genomic basis of olfaction might show signatures of adaptive or diversifying selection within these two groups.

In addition to finding food resources, most social communication among bats is also often focused on auditory cues. There is evidence of population divergence and sexual dimorphism in acoustic calls that may contribute to conspecific identification and speciation (e.g., Dávalos et al., 2019). However, there are a plethora of species-specific studies that often describe chemically-mediated social behaviors in bats, including pup scent-marking (Gustin & McCracken, 1987; Loughry & McCracken, 1991), mate courtship (Murray & Fleming, 2008), and establishing social hierarchy (Bouchard, 2001, 2005; Dechmann & Safi, 2005; Vaughan & O'Shea, 1976). As these behaviors extend beyond the olfactory-reliant pteropodids and phyllostomids, it suggests that chemical social communication in bats is widespread across most bat families. There are numerous reports of large sebaceous glands (Flores et al., 2019; Safi & Kerth, 2003; Scully & Fenton, 2000; Tandler et al., 1997), ranging from above the eyebrows, to the chest, to under the wing, which often have an unknown purpose; yet these large anatomical structures suggest that chemical secretions are somehow essential to the functional ecology of the animal.

The study of chemosensation is challenging, as it is complex relative to other senses (Yohe & Brand, 2018). The signaler releases complex chemical bouquets composed of dozens of compounds that often must be of a particular concentration to be detected (Figure 1), which then must be distinguished against a chemical background in the surrounding environment. These chemicals are then deciphered by the detector, which is performed by receptors encoded by large gene families—in fact, the largest and fastest-evolving gene families in the genome (Yohe, Fabbri, et al., 2020)—which are expressed in a complex labyrinth of epithelia, ducts, and nasal bones (Figure 1). The scale at which chemoreceptors duplicate in mammalian genomes vastly exceeds what is observed in insect models by orders of magnitude (Nei & Rooney, 2005), presenting a significant challenge even in rodent models or humans to determine which receptors bind to which compounds—a process known as "deorphaning" (Touhara, 2007). To add an additional layer of complexity, a second chemosensory system exists in the mammalian nose—the vomeronasal system—and initial signaling begins in an organ located at the anterior region of the nose (Figure 2). This narrow organ of neural epithelial tissue is supported by vomeronasal cartilage structures and has similar chemosensory receptor mechanisms as the main olfactory system, but with a distinct set of gene families. The vomeronasal system has been largely assigned to detect pheromones, or social chemical cues that mediate behaviors such as parental care, territoriality, and mating or courtship behaviors (Silva & Antunes, 2017; Tirindelli, 2021). Most of this knowledge, however, is evidence exclusively from rodent models. The focus of this paper is to synthesize what is known of these two systems, at both the genetic and morphological level, in the highly diverse mammalian group of bats.

Given the extensive variation in behavior and ecology within bats, there is a natural inclination to predict that the chemosensory anatomy and genes may reflect ecological adaptation. However, the evidence supporting this narrative is turning out rather nonintuitive—we are only beginning to scratch the surface of understanding the patterns emerging in bat chemosenasation. In a series of studies led by Kunwar Bhatnagar and

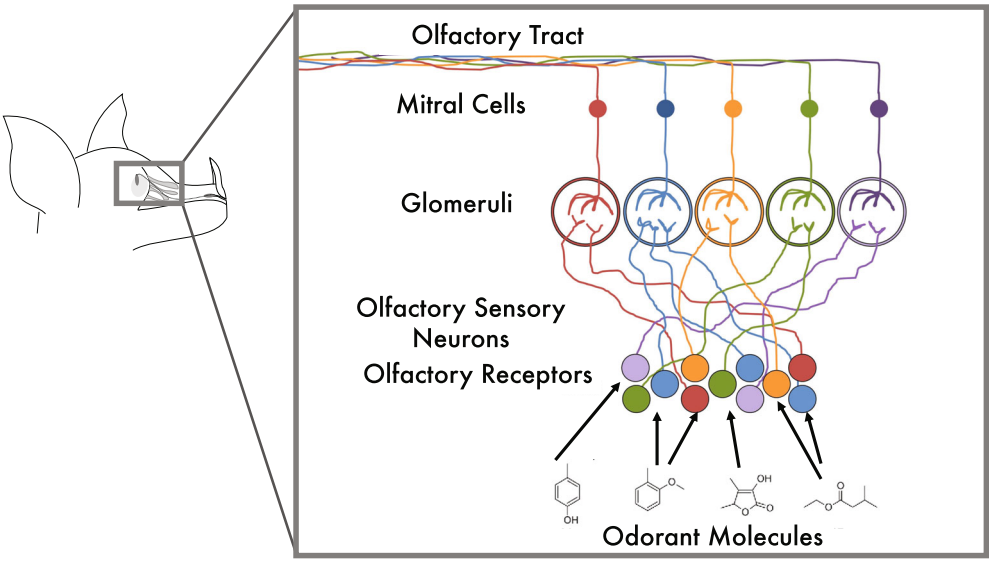

FIGURE 1 Mechanisms of olfactory signaling in terrestrial mammals. Bouquets of odorant compounds are inhaled into the nasal cavity and bind to olfactory receptors in a combinatorial fashion. Each olfactory sensory neuron expresses a single gene copy. Sensory neurons expressing the same alleles converge onto the same glomerulus cell in the olfactory bulb, and then further cascade into their respective mitral cells to be processed.

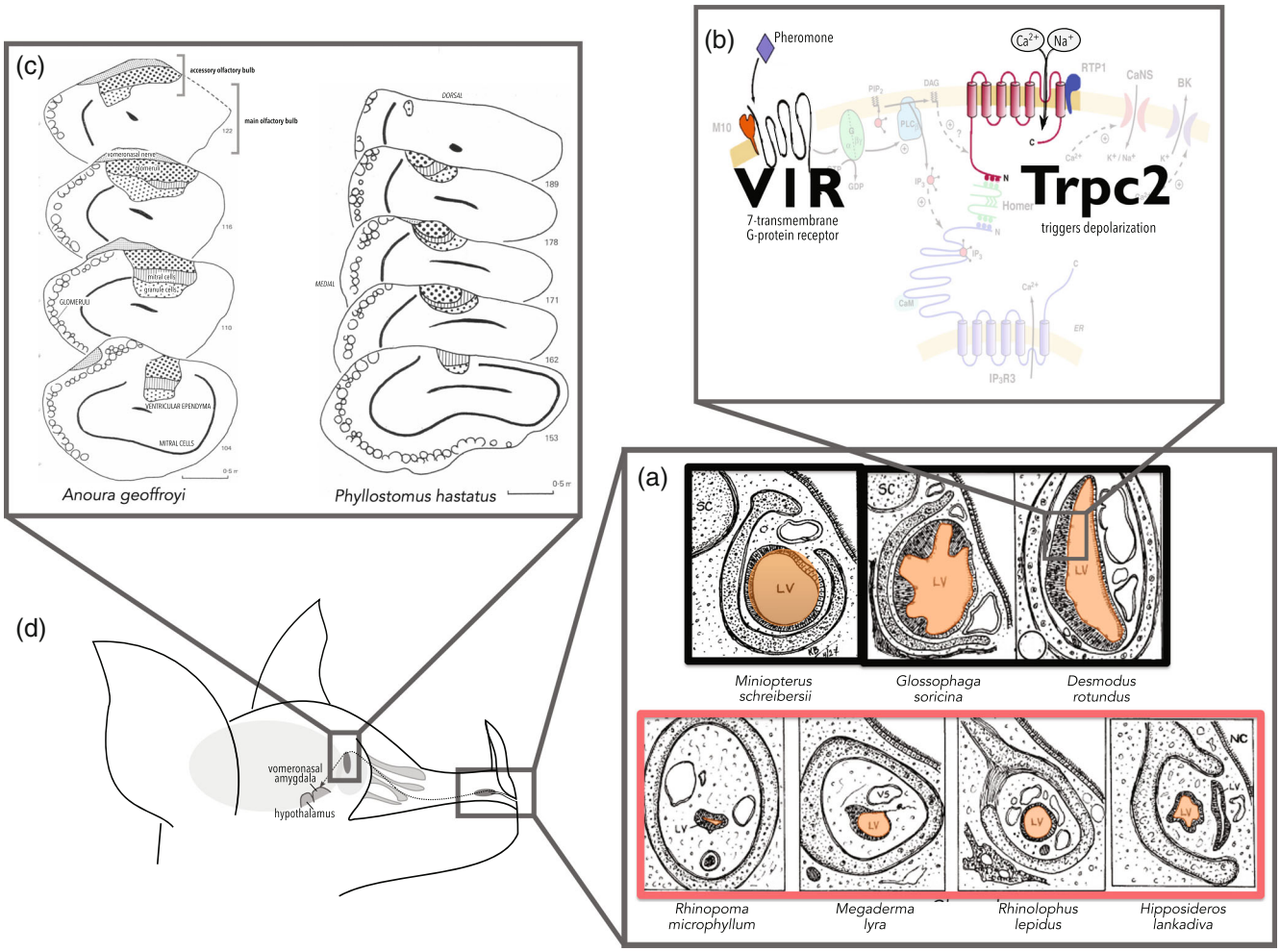

FIGURE 2 Components of the bat vomeronasal system. (a) Tracings of the vomeronasal organs from several bat species. The top row shown in black shows two clades (Miniopteridae, left; Phyllostomidae, right) that have retained a well-developed vomeronasal organ, though with extensive variation in structure. The bottom row in pink shows species with rudimentary vomeronasal organs. LV, lumen of the vomeronasal organ (highlighted in orange); NC, nasal cavity; SC, septal cartilage; VS, vascular sinus. Adapted from Bhatnagar and Meisami (1998). (b) Cell signaling pathway of mammalian Vomeronasal-type 1 receptor (V1R) signaling. Pheromone chemical compounds dissolved in fluid in the vomeronasal lumen bind to V1R receptors expressed in the neuronal epithelial tissue of the vomeronasal organ. V1Rs are G-protein-coupled receptors and when a conformational change is induced from the pheromone-binding, it triggers a signaling cascade that activates the Transient receptor potential cation channel 2 (Trpc2), which opens and enables calcium to flow into the cell and depolarize the neuron. Figure adapted from Mast et al. (2010). (c) Schematic tracings of thin sections of the accessory olfactory bulb in two phyllostomid species. The textured layers represent the accessory olfactory bulb, which is nested within the main olfactory bulb. Capital letters indicate layers of main olfactory bulb and lowercase letters represent accessory olfactory bulb layers. Modified from Frahm and Bhatnagar (1980). (d) Input from the accessory olfactory bulb subsequently connects directly to both the vomeronasal amygdala and the hypothalamus. Modified from Yohe et al. (2017).

colleagues, we begin to gain a sense of the curious patterns that have emerged to enable evolutionary hypothesis testing (Bhatnagar & Kallen, 1974a, 1974b, 1975; Bhatnagar & Meisami, 1998; Frahm & Bhatnagar, 1980; Reep & Bhatnagar, 2000; Smith et al., 2012; Wible & Bhatnagar, 1996). Considering new genetic and genomic evidence that has both updated the phylogenetic history of bats and illuminated molecular signatures of diversification and loss throughout bat history, it is necessary to revisit the myriad of diverse data sets of the nasal anatomy detailed by Bhatnagar and colleagues to reframe hypotheses moving forward.

2 | GENERAL ANATOMY AND GENETICS OF MAMMALIAN CHEMOSENSATION

To help orient the reader on the multiple components of the mammalian nasal chemosensory systems, we have

outlined below some general descriptions of the morphological and molecular proxies used to study the evolution of its diversity.

2.1 | From histology to diceCT: Morphological basis of chemosensation

The nasal cavity has two primary functions in the mammalian nose: chemosensation and respiration. As air enters, it first interacts with the respiratory epithelium, through which it is warmed, gas is exchanged, and foreign contaminants are filtered and cleared. This epithelium tends to be quite glandular in nature due to mucus excretion and is distributed along the walls of the anterior region, though especially along the ventral-medial areas (Pang et al., 2016; Smith et al., 2020; van Valkenburgh et al., 2014). It is also distributed along several turbinal bones (innervated and vascularized thin bones that extend posterior to anterior coated in epithelial tissue), but most commonly the maxilloturbinals. While the focus of this paper is the chemosensory systems, the distribution of respiratory epithelia is worth mentioning as it is often thought of as a tradeoff in terms of nasal cavity space, with more "olfactory ability" being devoted to a nose with more a higher proportion of olfactory epithelia (Martinez et al., 2020). As we move more posterior into the nasal cavity, we enter a labyrinth of bony scroll-like or thread-like structures that are the ethmoturbinals, usually coated with olfactory epithelium (Figure 1). The naming for these bones has been quite inconsistent through time (e.g., endo- and ectoturbinals in Bhatnagar & Kallen, 1974b, 1975; front-, inter-, and ethmo- in Yohe et al., 2018, 2022 with different numbering systems), and we will use terminology outlined in a more recent study (Yohe et al., 2018). Furthermore, for those less familiar with nasal anatomy, turbinate and turbinal can be used interchangeably but we will use turbinal henceforth. This anatomy is hypervariable (Bhatnagar & Kallen, 1975), and determination of the homology is challenging. Developmental studies will help to inform bones that may be truly homologous to one another across more disparate species (Ito et al., 2021).

The secondary chemosensory system, the vomeronasal system, sits anterior in the nose (Figure 2a). The entrance to this organ is not through air contact in the nostril, but rather the organ is thought to detect nonvolatile chemical cues, requiring physical contact with the chemical compounds to induce detection. The rodent model for pheromone detection is described here. Mucus-dissolved pheromonal compounds are delivered to the vomeronasal organ via vomeronasal ducts that open into the mouth, which presumedly pump the fluid to the lumen, an opening of the organ that is coated in vomeronasal sensory neuroepithelium (Figure 2a; Tirindelli, 2021). The mucus is then cleared through the nasopalatine ducts in the posterior region of the palate into the mouth. In most mammals, there are two layers of vomeronasal epithelia: $G_{\alpha i2}$-expressing and $G_{\alpha o}$-expressing neurons that mono-allelically express vomeronasal Type-1 receptors (V1R) and vomeronasal Type-2 receptors (V2R), respectively (Silva & Antunes, 2017; Stowers & Kuo, 2015). The vomeronasal epithelia and shape of the lumen in bats is highly variable (Figure 2a), with some species demonstrating highly deflated and poorly developed epithelia and others showing well-structured neurosensory epithelia. Regardless, there only appears to be a single (likely $G_{\alpha i2}$-expressing) neuroepithelium among the vomeronasal-possessing bats (Cooper & Bhatnagar, 1976).

At the level of the brain, the innervation from the nose to the bulbs is similar for both systems. The olfactory sensory neurons continue to thread through tunnel-like regions in the postero-dorsal nasal cavity (Levy et al., 2020) and eventually onto the cribriform plate of the ethmoid bone, where the neurons converge and onto their respective glomeruli of the olfactory bulb (Figure 1). In rodents and other mammals, there is a dimorphism in the accessory olfactory bulb, with the anterior region receiving input only from vomeronasal $G_{\alpha i2}$-expressing neurons and the posterior region receiving the $G_{\alpha o}$-expressing neurons (Tirindelli, 2021). There is no evidence of a posterior region in bats, despite the layering being quite complex and variable in nature (Figure 2d). Both the olfactory bulb and the accessory bulb will synapse onto distinct amygdaloid nuclei (Reep & Bhatnagar, 2000). Beyond amygdala volumes (Baron et al., 1996), inspection of distinct nuclei and the afferent neuronal projections in bats is not one that has been explored, especially in the context of olfaction.

There are several morphological proxies used to estimate "olfactory ability," including olfactory bulb size (e.g., volume), olfactory epithelium surface area (Eiting, Smith, & Dumont, 2014; Yohe et al., 2022), and olfactory bulb ratio (maximum linear dimensions of the olfactory bulb and the cerebral hemisphere in endocranium) (Zelenitsky et al., 2009). None are truly known to demonstrate olfactory ability, and inconclusive correlations with one another make it difficult to determine what morphological proxy is best reflective of olfactory reliance (Eiting, Smith, & Dumont, 2014). Bhatnagar has demonstrated that perforation of the cribriform plate on the ethmoid bone might relate to dietary ecology with an extensive data set (Bhatnagar & Kallen, 1974a), and cribriform plate morphology has some intriguing molecular correlates in mammals (Bird et al., 2018). In addition to the cribriform plate, the olfactory bulb ratio has promising behavioral and molecular correlates, particularly in

archosaurs. These patterns also make it useful to make inferences in extinct taxa as well (Zelenitsky et al., 2009, 2011). This has not been investigated in bats in a phylogenetic quantitative framework since its description and warrants further investigation as use of a proxy.

Much of the detail in the nasal cavity requires understanding of fine-scale transitions between epithelial layers and noting small organ structures that require serial sectioning, especially for vomeronasal structures that are vestigial in nature. It would be quite easy to miss these structures had it not been for the devotion of Bhatnagar to ensure the histology was done at fine scales (Smith, 2023). The advancement of micro-computed tomography (μCT)-scanning has transformed how we understand how the complex morphologies of the nasal cavities are oriented in space. Staining of fixed specimens using diffusible iodine-based contrast-enhanced CT-scanning (diceCT) enables the fine-scale visualization of how the serial histology "fits together" (Gignac et al., 2016). These diceCT methods can even be applied to bat embryos (Ito et al., 2021). DiceCT has advanced our understanding of bat nasal anatomy in several ways. First, resolution of olfactory and respiratory epithelial tissue is distinguishable with diceCT, facilitating our understanding of boundary transitions across turbinals in three dimensions (Yohe, et al. 2022). This distinction, which had been previously challenging given the overlap of both tissue types on a single turbinal, is important for developing airflow models and understanding odorant deposition, gas exchange, and thermoregulation. Second, diceCT allows the investigator to identify and reconstruct soft tissue structures, such as the olfactory epithelial tissue and vomeronasal organ, without severely sampling the specimen in destructive ways. This enables studies to scale up taxonomic sampling in feasible ways, and it has proven useful as a means for understanding the rampant vestigialization patterns of the vomeronasal organ across bat taxa. While this new imaging technology has greatly enhanced anatomical studies, it is often best to complement the 3D data with histological correlates to get a detailed understanding of the cellular and tissue-specific functions for regions of interest (Smith et al., 2020, 2022; Yohe et al., 2018). μCT- and diceCT-scanning also enables the compilation of large comparative datasets without significant damage to specimens, which gives more statistical power when trying to infer evolutionary patterns using phylogenetic comparative methods.

2.2 | From single loci to the largest mammalian gene families: Genomic basis of chemosensation

Olfactory receptor (OR) genes are a highly diverse clade of 900 intron-less base-pair genes that encode G-protein-coupled receptors that are mono-allelically expressed in olfactory sensory neurons but bind odorant cues in a combinatorial and somewhat promiscuous fashion (Figure 1; Niimura, 2012, 2013; Niimura et al., 2018). Within mammals, there are two major subfamilies of *OR*s that are determined by conserved binding motifs, known as Class I and Class II genes. It is frequently stated that Class I receptors are associated with detecting water-borne odorant compounds while Class II receptors are predicted to detect volatile airborne cues (Hayden et al., 2010), but this mostly is inferred from the large expansion of Class II in mammals and has never functionally been demonstrated. These genes, as well as vomeronasal receptor genes, evolve via birth–death dynamics (Nei & Rooney, 2005). To put it simply, this means these genes duplicate and either accumulate a novel beneficial mutation and neofunctionalize or accumulate a mutation such as a premature stop codon that would render the gene nonfunctional (pseudogenize). There are deeper characterizations of fates of gene duplicates and classifications into more precise subfamilies (Yohe, Liu, et al., 2019), but we will characterize the genes as "intact" or "pseudogenized" henceforth. Other reported gene families associated with chemosensation (such as trace amine-associated receptors [*TAAR*s], Eyun et al., 2016 and *formyl peptide receptors* [*FPR*s], Bufe et al., 2015; Liberles et al., 2009; Rivière et al., 2009), but are much lesser understood and not considered here (though certainly worthwhile and necessary to explore in bats). Bat1K, the genomics consortium with the initiative to sequence all bat genomes to chromosome-level assembly (Teeling et al., 2018), has greatly facilitated chemosensory gene discovery. However, these regions are so duplicated and so variable that our understanding of the diversity in humans is even lacking given the difficulty to resolve segregating alleles in orthologs from recently duplicated paralogs. By far the most common molecular proxy of "olfactory ability" is the number of intact olfactory gene copies in the genome or proportion of intact versus pseudogenized genes (which is a form of counts) (Hayden et al., 2010, 2014; Hughes et al., 2018; Tsagkogeorga et al., 2017; Wang et al., 2020). However, we outline below why this is very misleading and biased, and often inappropriate, and that rates of evolution in these genes may be more informative to understand sequence diversity and selection.

The receptors of the vomeronasal system are similar to the olfactory system in terms of mechanism but are encoded by different gene families. The *V1R* and *V2R* gene families are highly diverse across tetrapods (Silva & Antunes, 2017; Young et al., 2010), and evolve via a similar birth-death manner as olfactory receptors. Note, *V1R*s are intronless like *OR* genes, but *V2R*s have more

complex gene structures and are composed of several exons (Tirindelli, 2021). Intriguingly, *V2R*s do not appear to be present in bats and show a low diversity in Laurasiatheria in general (Dinka et al., 2015; Grus, 2008; Grus et al., 2005; Grus & Zhang, 2006, 2009). When a *V1R* is activated, it triggers the G-protein signaling cascade that facilitates the membrane-bound ion channel *Transient potential receptor cation channel 2* (*Trpc2*) to open and trigger depolarization of the neuron (Mast et al., 2010) (Figure 2b). Most species that tend to have vestigial elements of a vomeronasal system also have absent or pseudogenized *Trpc2*, *V1R*, and *V2R* genes in their genome, making a decent proxy for vomeronasal function (Kishida et al., 2015; McGowen et al., 2008; Yohe et al., 2017; Yu et al., 2010; Zhao et al., 2011). Because of the duplicative nature of *V1R* and *V2R* genes, they run into similar issues observed in the olfactory system in terms of trying to assign functional meaning to gene copy abundances. There is a single ortholog of the *V1R* gene family, known as *ancV1R*, that is present in all vertebrates and promiscuously expressed in vomeronasal neurons (Suzuki et al., 2018). Pseudogenization or absence of this gene, like *Trpc2*, is also highly correlated with vomeronasal morphological vestigialization (Zhang & Nikaido, 2020) and may also serve as a proxy of function. Note that while these molecular sources of data may be useful proxies, direct functional relationships of olfactory and pheromone-detecting abilities and molecular patterns (e.g., number of chemoreceptor genes) are rarely demonstrated outside of model organisms and must be interpreted with caution.

3 | MATERIALS AND METHODS

Much of the data and material presented in this synthesis is derived from the literature. To provide the most up-to-date descriptions of molecular diversity of chemoreceptors in bats, we applied our previously published pipeline (Yohe, Fabbri, et al., 2020) to all available bat genomes to date (see Supporting Information Table S1 for genome versions). The pipeline is described briefly here. Amino acid sequences of tetrapod *OR* genes and vomeronasal receptor genes (Types 1 and 2) were blasted against published bat genomes using blast version 2.11.0 with the tblastn algorithm (Gerts et al., 2006). Blast was configured to report the top 1000 highest-scoring matches to ensure all duplicates were retained. The blast output was then parsed using genomeGTF tools *blast2gff* version 1.2 (https://github.com/wrf/genomeGTFtools). The resulting .gff3 file was then converted into a .fasta file using bedtools2 *getfasta* version 2.29.0. All output from each genome was run through bbmap *dedupe.sh* version 38.94 to remove all genes that were exact duplicates or containments (sourceforge.net/projects/bbmap/). The remaining genes were run through a modified olfactory receptor assigner (ORA) version 1.9.1, which had been retrained on a custom dataset to allow for the classification of *ORs* and vomeronasal genes (Hayden et al., 2010). *ORA* is a perl script that implements a hidden Markov model to assign each gene to a subfamily based on conserved sequence motifs and classifies genes as pseudogenized or intact depending on whether the reading frame possessed a premature stop codon mutation, frameshift mutation, or was less than 650 base pairs. The classified genes were then run through *dedupe.sh* once more because ORA can shift the reading frame. The final output was parsed into count tables using a custom python script. All harvested chemoreceptor gene sequences are available as supplemental material.

4 | THE EVOLUTION OF THE OLFACTORY SYSTEM IN BATS

4.1 | What we have learned

4.1.1 | Extensive variation in turbinal and olfactory bulb morphologies

Bhatnagar and colleagues observed differences in olfactory epithelium among species, mostly interpreting patterns based on diet and phylogeny (Bhatnagar & Kallen, 1974b, 1975), including expanded surface areas and thicker epithelium in frugivores. Intriguingly, the turbinal morphology within pteropodids has been described as "unspecialized" (e.g., *Rousettus leschenaultii*; Smith et al., 2021), and relatively similar to what is observed in other mammals. On the other extreme, rhinolophids have exceptionally unusual turbinal morphology: both the ethmo- and maxilloturbinals have been modified in a strand-like structure (but see Ito et al., 2021), potentially to facilitate nasal echolocation transmission or tuning (Curtis & Simmons, 2016). Rhinolophids demonstrate some of the most exceptional high duty cycle echolocation calls within bats (Jones et al., 2013), and we propose that natural selection may have potentially "highjacked" the turbinal system to enhance more sophisticated acoustic transmission. Within a monophyletic clade of Caribbean nectarivorous phyllostomids, *Brachyphylla cavernarum* has evolved an extra, large interturbinal coated in olfactory epithelium (Yohe et al., 2018). Why only *Brachyphylla* and not closely related *Erophylla* or *Phyllonycteris*? We have virtually no sense as to how turbinates vary at the population level within a species, and this would be a natural next step to begin to interpret derived variation in closely related species.

It is a well-established trend that the olfactory bulb is larger in both radiations of plant-visiting bats (Barton et al., 1995; Reep & Bhatnagar, 2000), but studies of Bhatnagar that have detailed the variation of serial sections of the olfactory bulbs that warrant a deeper look (Bhatnagar & Kallen, 1974a; Frahm & Bhatnagar, 1980). Often the outlier examples in Bhatnagar's studies were "ahead of their time" and decades later molecular work has supported hypotheses he stated deep within a paragraph in a discussion. For example, the neotropical insectivorous mormoopid *Pteronotus rubiginosus* (formally, *Chilonycteris rubiginosa* at the time of publication) was pointed out that the olfactory bulb size and cribriform plate structure as the more derived phyllostomid clades, and even posits that the ancestor of that superfamily may have been frugivorous (Cooper & Bhatnagar, 1976). This notion of at least a more omnivorous ancestor in the superfamily of Noctilionoidea has been supported by many recent molecular (Davies et al., 2020; Potter et al., 2021) and morphological (Hall et al., 2021) studies, such that selective signatures that would facilitate plant-visiting occurred much more basal to the evolution of plant-visiting in observed extant taxa. There are developmental (Camacho et al., 2019) and paleontological studies (Yohe et al., 2015) that support this hypothesis as well. Further investigations that leverage comparative phylogenetic methods that rigorously statistically demonstrate patterns of trait evolution will continue to unveil more meaningful evolutionary patterns throughout bat diversification.

4.1.2 | Olfactory receptor diversity is vastly misrepresented, but molecular diversity suggests most patterns are largely endogenous

There are several confounding issues with drawing conclusions from previously published *OR* gene data in bats. This is either due to methodological variation in the sequencing of these *OR* genes or outdated and poor-quality genome sequencing in which bioinformatics pipelines are applied to identify *OR* genes. More specifically, because early *OR* data was generated from PCR and cloning techniques using degenerate primers (i.e., non-specific primers that will amplify *OR* genes based on conserved membrane domains in an attempt to capture as much diversity as possible; Hayden et al., 2010, 2014), there are issues with both gene recovery and primer bias (Yohe, Davies, et al., 2020). Second, most published studies of bat *OR* genes use either published counts without a re-harvesting of the updated genomes or do not perform a comprehensive survey of the genome, especially as new ones have become available. Studies may even merge data from different studies where data was acquired with different methodologies and pipelines (e.g., Garrett & Steiper, 2014). Using an updated and consistent pipeline (Yohe, Davies, et al., 2020; Yohe, Fabbri, et al., 2020), the actual identified gene counts are sometimes nearly an order of magnitude higher, especially when considering pseudogenes (Figure 3b). For example, from the genome using our pipeline, *Carollia perspicillata* has a reported 1115 intact olfactory receptor genes (Figure 3b), a number that is a slight underestimate from what was obtained when sequenced from a different individual using targeted sequence capture (1148), but an extreme difference from the previously reported 190 receptors obtained from PCR (Hayden et al., 2014). It is critical to note here that in the previously published study for phyllostomids, the underrepresentation was presented in the supplement with targeted estimates (in fact, it was projected that *C. perspicillata* did indeed have 954 receptors), but all analyses and conclusions were performed with data of the severely underestimated counts (Hayden et al., 2014; Wang et al., 2020). Even relying on genomic data, the pipeline used in this paper that is reliably annotating across methods results in significantly higher counts than previously reported for several species: (*Pteropus giganteus*: 261 from Hughes et al., 2018; 1022, this study). A very similar pattern in inconsistency was reported for *Desmodus rotundus*. Yohe, Davies, et al. (2020) found 424 intact *OR* genes, where previous estimates from the genome were 135 (Hughes et al., 2018) and 212 genes from PCR cloning (Hayden et al., 2014). We report that as genome assemblies have improved, here we have found 649 receptors for *D. rotundus* than when we reported in 2020 (Yohe, Davies, et al., 2020). The counts here are the most accurate to date, though again should be considered with caution given issues with misassembly of these highly repetitive regions.

The purpose of detailing the issues with the genomes and *OR* counts above is to emphasize that the counts are not a reliable way to infer ecological or evolutionary adaptation. Detecting adaptation when orthology versus paralogy remains an outstanding challenge in molecular evolution and more developed gene duplication models must be developed for concluding that selection has occurred (Yohe, Liu, et al., 2019). Now that we have outlined the caveats of the currently available data with *OR*s, are there any meaningful patterns of adaptation that have come to light from these sequences? One approach is to characterize the rates of evolution in the sequences themselves. In phyllostomids, when looking at rates of evolution of nonsynonymous (amino-acid changing) substitutions relative to the rates of synonymous changes,

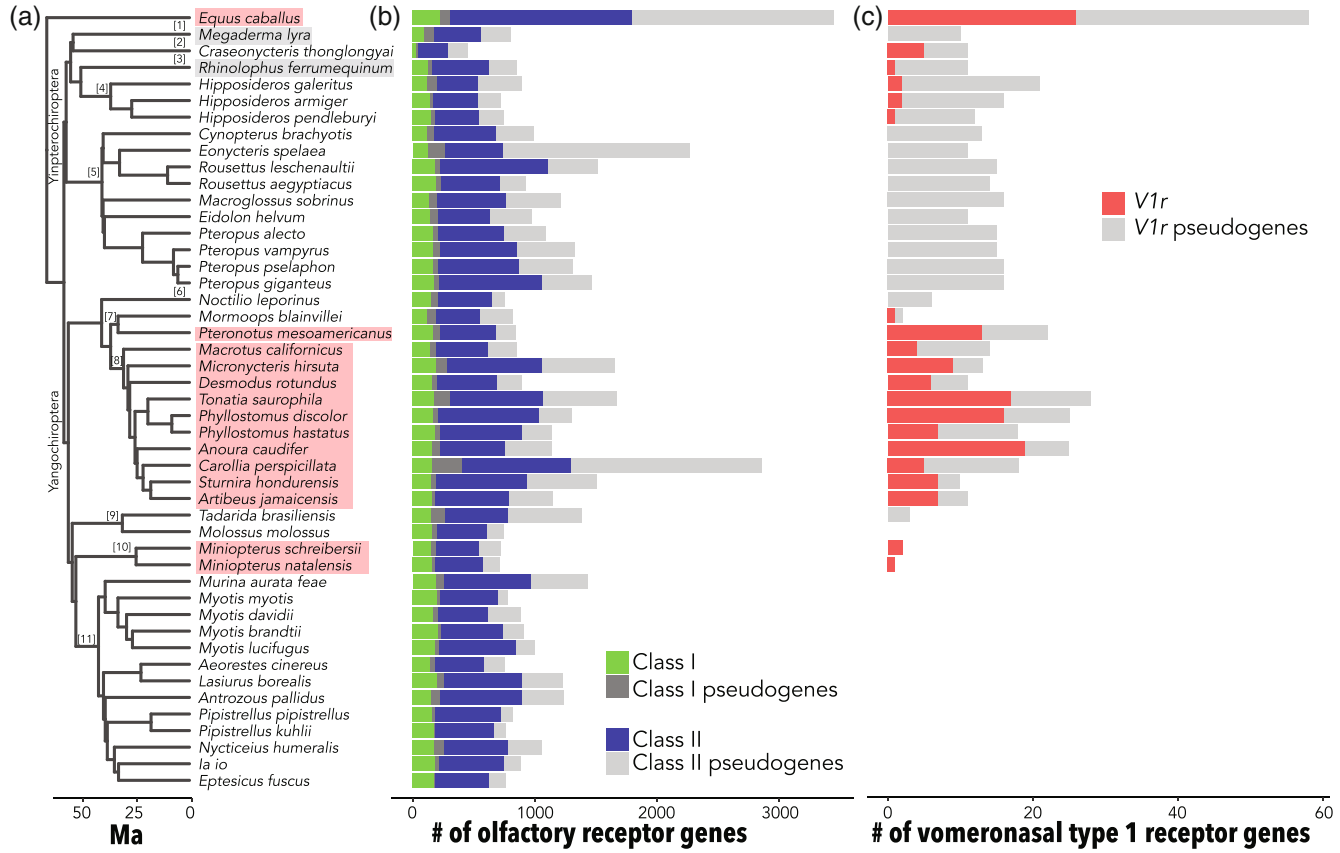

FIGURE 3 Chemosensory gene counts for bats inferred from the genomes. (a) Phylogeny of bats with genomes available on GenBank. Numbers correspond to major bat families: Megadermatidae (Jones et al., 2013), Craseonycteridae (Page & Hofstede, 2021), Rhinolophidae (Burgin et al., 2018), Hipposideridae (Nesi et al., 2021), Pteropodidae (Rojas et al., 2016), Noctilionidae (Shi & Rabosky, 2015), Mormoopidae (Gonzalez-Terrazas et al., 2016), Phyllostomidae (Hodgkison et al., 2013), Molossidae (Korine & Kalko, 2005), Miniopteridae (Dumont et al., 2012), Vespertilionidae (Dávalos et al., 2019). Pink boxes indicate clades where most species have an intact vomeronasal system. Gray boxes are clades that have a vomeronasal organ but no accessory olfactory bulb. Tree is trimmed from a concatenated tree of Rojas et al. (2016) and Shi and Rabosky (2015). (b) Total number of intact and pseudogenized olfactory receptors (*OR*s) from each genome, binned into subfamilies of Class I and Class II. (c) Total number of intact and pseudogenized *V1R*s from all bat genomes. If no bar is displayed, no intact or pseudogenized gene was detected.

there was no difference observed in animal-feeding versus plant-visiting species (Yohe et al., 2022). Rather, many Class I genes were found to be evolving at higher rates (diversifying or relaxed selection) and some subfamilies of Class II genes seemed to have exceptionally low rates (i.e., purifying selection), independent of diet (Yohe et al., 2022). Another approach is to incorporate the evolutionary history of duplications since speciation (Hughes et al., 2018; Yohe et al., 2021). At shallower scales, for example, within the phyllostomid genus *Carollia* where conspecific and co-occurring species demonstrate a spectrum specialization to *Piper* plants, the more specialist species had a narrower diversity of *OR* genes compared to the generalist, which may reflect a more fine-tuned olfactory profile to locate a more specific food resource (Yohe et al., 2021). However, we have virtually no understanding of what any of these receptors bind to, as no receptors have been deorphaned for bats, thus all ecological interpretations of these patterns are largely speculative. Analyses of rates of duplication and loss have also been measured within bats in a sample of eight species; a general contraction in *OR*s was noted at the divergence of bats from other Laurasiatheria and an expansion of *OR*s was detected in pteropodids (Tsagkogeorga et al., 2017). But again, these studies require inputs of counts and can be highly biased. Furthermore, measuring the rate of duplication and loss is suggesting the notion that duplication is not a stochastic phenomenon (which it is), and thus should be relatively constant through time. Rather studies should be measuring rates of gene retention (Yohe, Liu, et al., 2019) that demonstrate an estimate of selection on duplicate genes.

4.2 | What we still do not know

4.2.1 | Spatial organization of *ORs* and additional gene family expression

Qualitatively, Bhatnagar remains the first to report on the enlarged and well-developed olfactory epithelium of a non-insectivorous bat, the neotropical and frugivorous *Artibeus jamaicensis* (Bhatnagar & Kallen, 1975), emphasizing that not all bats have reduced olfactory morphology in their nasal cavities. However, when correcting for phylogenetic signal, there was only weak predictive power in the surface area of plant-visiting in yangochiropterans (Yohe et al., 2022) and very little correlation with epithelial surface and reliance on olfaction (i.e., plant-visiting; Eiting, Smith, & Dumont, 2014). It was only after the rates of evolution of the *OR*s were analyzed with the morphological data that ecological signatures of adaptation were detected, and the relationship was inverse (Yohe et al., 2022). Essentially, *OR* genes evolved more slowly (i.e., were under functional constraint) in plant-visiting bats with larger surface areas of olfactory epitheliums, and this relationship was only detectable when the molecular and morphological data were considered simultaneously. In addition to suggesting ecological constraints on hyper-evolving *OR*s, it also suggests a more complex relationship between olfactory surface areas and *OR* gene expression. Are plant-visiting bats expressing particular subfamilies of *OR*s, perhaps the receptors under strong purifying selection, at higher rates? What role does the shape of the turbinal play in expanding and enhancing detection? Do certain turbinals only express certain subfamilies? We are in a new frontier in unveiling how the genomics and morphology of such a complex sensory system are intertwined: rodent models are developing the methods and techniques to map the molecular landscape of the nasal cavity (Coleman et al., 2019; Noto et al., 2017; Tan & Xie, 2018). Performing similar applications in bats would unveil what variation in turbinal shape and size means functionally, and connecting patterns with airflow models will aid in understanding odorant inhalation, deposition, and detection (Eiting et al., 2015; Eiting, Smith, Perot, & Dumont, 2014).

Additionally, genomic characterization and gene expression of other chemosensory gene families (*FPR*s, *TAAR*s) are poorly described in bats, but their relation to detecting food resources or even pathogens is another intriguing avenue. The large variation in the number of *TAAR*s in the two bats (ranging from 6 in *Myotis lucifugus* to 26 in *Pteropus vampyrus*) that have been investigated is relatively higher than other mammals and this gene family might be an intriguing source of diversity to explore, especially with the onset of new genomes (Eyun et al., 2016). It is worthwhile to reiterate that both molecular rates of evolution of chemoreceptors and the numbers of chemoreceptors do not have clear implications on "olfactory capabilities," and one must caution the interpretation of these rates without functional demonstration of olfactory performance.

4.2.2 | Further representation of bat families

Outside of Phyllostomidae and a few other yangochiropterans, there is little data of both the high-quality molecular *OR* gene sequencing or soft tissue diceCT scans, especially made publicly available. Given that Pteropodidae represents an independent and convergent radiation of plant-visiting, this provides a prime opportunity to explore parallel gene family expansions, *OR* expression along turbinals, overall olfactory and respiratory distribution, and olfactory bulb innervation. Evidence of convergent trends among pteropodids and phyllostomids will facilitate knowledge in the field of chemosensation more broadly, as there are very few mammalian clades that demonstrate the evolution of divergent diets as bats do, and it will help advance understanding of whether finding food resources select for specialized sensory systems. Figure 3b demonstrates a diverse range of *OR* repertoires across all clades and the evolutionary dynamics among other clades beyond plant visitors. The unusual olfactory turbinate rearrangements identified from bony μCT-scans in Rhinolophidae offer an opportunity to connect whether this came at the cost of particular *OR* gene subfamilies while optimizing for nasal phonation (Curtis & Simmons, 2016).

5 | THE EVOLUTION OF THE VOMERONASAL SYSTEM IN BATS

5.1 | What we have learned.

5.1.1 | Bats have retained ancestral vomeronasal function, while many clades have independently lost function

Through several phylogenetic mapping studies of the vomeronasal system anatomy by Bhatnagar and colleagues, it was posited that the most parsimonious scenario of vomeronasal system evolution is that it was lost ancestrally in bats and has been regained in the few families or species that have function (Bhatnagar & Meisami, 1998; Wible & Bhatnagar, 1996). Genetic evidence from *Trpc2* and *V1R*s have helped to clarify this and

support the less parsimonious scenario, such that the ancestral bat had an intact vomeronasal organ and it has been subsequently lost many times in bats (Yohe et al., 2017; Yohe, Davies, et al., 2019; Zhao et al., 2011). *Trpc2* has many derived pseudogenizing mutations, sometimes shared at deeper divergences of bat families, and some recently derived at the species level (Yohe et al., 2017; Zhao et al., 2011). Simulation experiments of *Trpc2* evolution show that selection to maintain function of this gene was strong in the ancestor of bats, and has subsequently lost function (Yohe et al., 2017). Bats with intact vomeronasal organs also have intact copies of *V1R*s in their genomes, and the copies have mostly been retained since bat-carnivore-ungulate divergence (Yohe, Davies, et al., 2019). Furthermore, species that had absent vomeronasal organs had pseudogenized copies of these orthologs. The confounding issues associated large gene families when studying the *V1R* gene family persist in mammals but given the limited diversity in bats this is not as much of a concern in terms of sequencing, assembly, and annotation. They can serve as a tool to study broader genomic mechanisms in bats. Intriguingly, nearly all vespertilionid bats have zero copies of either intact or pseudogenized *V1R*s (Figure 3c). The absences of these corroborates anatomical data, but why have all evidence of psuedogenes been lost? Studying the gene family dynamics of these chemosensory systems can unveil additional insight on genomic rearrangement and loss through bat evolution, as well.

5.1.2 | Several bat clades have lost vomeronasal function relatively recently

From the shared pseudogenizing mutations of a number of bat families (Yohe et al., 2017; Zhao et al., 2011) and close association with *Trpc2* pseudogenization and an absent accessory olfactory bulb (Yohe & Dávalos, 2018), it can be inferred that vestigialization of the vomeronasal system did occur deeply in the ancestor of most clades with absent variation. However, there are a number of species that appear to have lost function of *Trpc2* more recently (Yohe et al., 2017; Yohe & Dávalos, 2018). Caribbean nectarivores (Brachyphyllini), for instance, are nested within Phyllostomidae, which are one of the few major clades to have retained vomeronasal function in all components of the system. However, these species have both a shared ancestral *Trpc2* pseudogenizing mutation (Yohe et al., 2017) and absent vomeronasal organ morphology (Yohe et al., 2018). Likewise, *Choeroniscus godmani*, another non-Brachyphyllini phyllostomid nectarivore has an absent accessory olfactory bulb (Frahm & Bhatnagar, 1980), and while the *Trpc2* is intact (Yohe et al., 2017), it appears the selective constraints have relaxed on the gene and it might be "on its way out" (Yohe & Dávalos, 2018). For what evolutionary reasons has selection suddenly shifted on this system in some phyllostomids and not others, after being maintained for tens of millions of years throughout bat evolution?

5.1.3 | Vestigialization is decoupled from ecological signatures

Perhaps what has remained most puzzling about the vomeronasal system in bats is why some bats have retained the functioning system and why some bats have lost complete function. Sifting through discussions of observations in Bhatnagar papers and combining these notes with observed trends in the molecular data (Bhatnagar & Meisami, 1998; Cooper & Bhatnagar, 1976; Reep & Bhatnagar, 2000; Wible & Bhatnagar, 1996), there appears to be no obvious ecological explanation for loss within different clades of bats. Shifts in diet, alternative sensory modalities (e.g., high-duty frequency echolocation, thermosensation), and social structure are decoupled from all evolutionary moments of "loss." Likewise, many species that lose function retain specialized behaviors relying on pheromones (Murray, 2008; Murray & Fleming, 2008). We do not know why some bats do or do not have a vomeronasal system, but we do know it is not related to general shifts in ecology or behavior. The data challenge our entire understanding of the purpose and function of the vomeronasal system, as well as the evolutionary constraints on craniofacial and sensory evolution, in general.

5.2 | What we still do not know

5.2.1 | Genomic and morphological mismatches

In Old World Yinpterochiroptera, there is a general assumption of extensive loss of function, vestigialization, and pseudogenization. For many taxa, this is certainly the case. However, if we are to turn deep into the anatomical descriptions by Bhatnagar, there is an abundance of very curious patterns of morphological retention and near or completely near loss of genomic evidence. *Megaderma lyra* appears to have a well-developed vomeronasal organ, a number of gland ducts and vasculature (Cooper & Bhatnagar, 1976; Smith et al., 2012), but an absent accessory olfactory bulb (Cooper & Bhatnagar, 1976), disrupted *Trpc2* (Zhao et al., 2011) and only pseudogenes remaining in its *V1R* genes (Figure 3c).

Genomic evidence of *Craseonycteris thonglongyai*, for which no anatomical data are known, has five intact copies of *V1R*s, comparable to some phyllostomids (Figure 3c). Has this curious monotypic species of the family and perhaps the world's smallest mammal retained the function of its vomeronasal organ despite pervasive loss in related families? As the genomic data of bats grows through Bat1K efforts, a similar pace of anatomical data must be systemically collected to interpret patterns and test hypotheses presented from molecular evidence.

5.2.2 | The new frontier of ducts, glands, and supporting structures

Nasopalatine and vomeronasal ducts are often noted as evidence for a vomeronasal organ (Jordan, 1972), but Bhatnagar points out the inconsistency in this conclusion in both bats and primates (Bhatnagar & Meisami, 1998). Recent evidence in mice has demonstrated that there are multiple "microtunnels" in the nasal cavity (Levy et al., 2020); these include perforations for neurons, cavities for ducts and fluid drainage, and vascularization by blood vessels. In some species, including *Rhinopoma microphyllum* and *Rhinolophus lepidus,* that are often dismissed as lacking a vomeronasal system, as they have pseudogenized *Trpc2* and lack an accessory olfactory bulb, there remains intriguing distinct structures and innervation patterns still observed in the organ (Cooper & Bhatnagar, 1976). Could it be that while most taxa have independently lost function of the organ in the ancestral mammalian vomeronasal system, some taxa have repurposed it for novel function? The use of diceCT complemented with histology has been highly informative of how innervation and vasculature of the nasal cavity are interwoven and connected (Smith et al., 2022), and will be informative in clarifying just how similar or different in function vomeronasal function may be across bats.

There are many more anatomical structures within the nasal cavity that still need to be better functionally described, and their understanding may facilitate clarification of what bats may be using their vomeronasal organs for. In rodents, small olfactory structures at the very anterior of the nose do not express chemoreceptors but project to a subset of olfactory bulb neurons called Grüeneberg ganglia (Roppolo et al., 2006). How well developed are these ganglia in bats and how do they differ in species with and without a vomeronasal organ? Miniopterids, for instance, are among the few chiropteran families to demonstrate a vomeronasal system with all the intact structures and genes (Figures 2 and 3), with no clear ecological reason as to why. They are also among the few bat families to have both mineralized elements in their nasal cartilage (Curtis & Simmons, 2018) and a rudimentary palatal branch of the premaxilla (Giannini & Simmons, 2007). Do these traits relate to retention of vomeronasal function in miniopterids? Furthermore, there are several bat species that have the intact organ without the ducts (Wible & Bhatnagar, 1996). How then do the pheromones get delivered to and cleared from the organ, if at all? And if it is not functioning to detect pheromones, what is the purpose of the organ? Finally, bats have highly variable external nasal morphology, described as nose-leafs. While much of their function may reside in facilitating echolocation, some structures are quite derived, and shape overall variation demonstrates association with bat ecology (Leiser-Miller & Santana, 2020). Given that nasal vomeronasal ducts protrude anteriorly in the snout and palate, the role of the nose leaf and nose-leaf innervation might somehow be related and warrants investigation.

5.2.3 | Behavior and the neurobiology of the accessory olfactory bulb

Though it resides completely nested within it, the accessory olfactory bulb is a distinct structure from the olfactory bulb that receives input from vomeronasal neurons and projects to distinct regions in the brain. Many bats have an absent accessory olfactory bulb, but the retention of pheromonal behaviors questions the simple hypothesis of "use it or lose it," and deeper comparative analyses of social chemical behavior in bats are much needed. *Tadarida brasiliensis*, for instance, a vespertilionid bat that lives in colonies sometimes by the millions, uses scent marking to identify its pups (Gustin & McCracken, 1987). Maternal recognition and care are well-known to be controlled by the accessory olfactory bulb and interaction and complementing the main olfactory system is necessary for behaviors such as accepting familial offspring; when disrupted these behaviors are drastically reduced in rodent female models (Keller et al., 2009; Lévy & Keller, 2009). How then is a species like *T. brasiliensis* identifying its pups? Has the connection to the medial amygdala from the accessory olfactory bulb been severed and rewired to only connect through the main olfactory bulb? Bhatnagar and colleagues have pondered this in several papers (Bhatnagar & Kallen, 1974a; Frahm, 1981; Frahm & Bhatnagar, 1980; Reep & Bhatnagar, 2000), but the question remains unanswered to date. It perhaps may not be just within bats, but a Laurasiatherian phenomenon (Keller & Lévy, 2012). But then what is happening in species with an intact accessory olfactory bulb? Expression of vomeronasal receptors and Trpc2-expressing neurons have been detected in the main

olfactory epithelium and olfactory receptors have been found in the vomeronasal organ in rodents (Ibarra-Soria et al., 2014; Omura & Mombaerts, 2014); this built-in redundancy may help to compensate for vestigialization of the organ and brain regions and warrants further investigation. As bat model systems emerge in neurobiology, a focus on scent behavior would help resolve some persisting questions. Brain atlases of *Phyllostomus hastatus* (Radtke-Schuller et al., 2020), *Carollia perspicillata* (Scalia et al., 2013), and *Pteronotus parnellii (*Washington et al., 2018) all exist, though the predominant focus has been auditory input and processing. The overall structure and layering patterns in the accessory olfactory bulb must more complex than just noting presence and absence, and appear very variable among species (Figure 2d; Frahm & Bhatnagar, 1980). A comparative analysis of the nuclei and their projections in olfactory-related inputs would be revealing in understanding how these neural circuits vary is warranted and has now become tractable as neuroimaging techniques have developed.

6 | CONCLUSION

An essential message that needs to be communicated to the broader realm of chemosensory research is that many bats have well-developed olfactory systems and many species very much have retained vomeronasal system function in intriguing and diverse ways. It must not be perpetuated that bats lack a vomeronasal organ or have poor chemosensory abilities, as it dismisses the opportunity to explore new mechanisms for system function not observed in other mammals. Given the morphological disparity in the structure, bats may even serve as an informative model system to better understand the often-misunderstood secondary chemosensory system in the nose. In broader comparative mammalian literature, bats are often binned in a general assumption that what is observed in one or two species is what represents the entire diverse clade. Bhatnagar and colleagues appreciated very early on that this, indeed, was not the case. Bhatnagar combined detailed single-species anatomical descriptions with large-scale phylogenetic datasets, sometimes comprising of over 30 genera. The foundations for forming novel and exciting hypotheses must turn to these patterns and connect them.

AUTHOR CONTRIBUTIONS

Laurel Yohe: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – original draft; writing – review and editing. **Nicholas T. Krell:** Data curation; methodology; software; writing – review and editing.

ACKNOWLEDGMENTS

Laurel R. Yohe would like to express gratitude to the editors for the invitation to contribute to this special issue for Kunwar Bhatnagar, whose research has had a profound effect on the direction of her career.

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How to cite this article: Yohe, L. R., & Krell, N. T. (2023). An updated synthesis of and outstanding questions in the olfactory and vomeronasal systems in bats: Genetics asks questions only anatomy can answer. *The Anatomical Record*, *306*(11), 2765–2780. https://doi.org/10.1002/ar.25290