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Skull morphological adaptations to acoustic emissions: peak frequency in bats

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Chiropteran species developed diverse sound emission systems (i.e. oral or nasal) which impose different structural and functional demands on the skull. Because peak frequency (FP) reflects key aspects of echolocation performance—such as spatial resolution, call attenuation and beam directionality—it provides a continuous, biologically meaningful proxy for sensory adaptation. To investigate how sensory adaptation influences cranial evolution, we examined the relationship between skull morphology (size and shape, quantified using three-dimensional geometric morphometrics) and FP across a macroevolutionary dataset representing approximately 65% of extant bat genera. Phylogenetic comparative methods were employed to assess skull morphological variation associated with FP. We predicted that species relying on multiple sensory strategies (e.g. frugivores) would exhibit weaker associations between skull morphology and FP compared to insectivorous species that depend more heavily on echolocation. Unexpectedly, we found that frugivorous nasal emitters presented significant skull shape (but not size)

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adaptations to the frequency emitted. In both insectivorous and frugivorous species, high frequencies were associated with a relatively short rostrum. Moreover, FP exerted stronger constraints on skull shape in nasal emitters than in oral emitters. Our results highlight FP as an important factor shaping skull morphology across bats, even in groups that rely on other sensory modalities.

1. Introduction

A variety of functional drivers can simultaneously influence the same phenotypic trait, often resulting in the evolution of complex adaptive systems or functional trade-offs [1–4]. The diverse design of mammalian skulls is an example of the adaptation to the different functional demands imposed by sensory and feeding functions [5–9]. Echolocating mammals use sounds as their main sensory system to navigate and pursue prey and therefore face physical acoustic demands on their head morphology (e.g. toothed whales' mandibles: [10]). Beside the allometric scaling of frequencies emitted by toothed whales and bats (i.e. the negative correlation between skull size and frequencies emitted [11,12]), little is known about the evolutionary constraints on cranial morphology imposed by echolocation.

Chiroptera evolved echolocation as an additional sensory system to perceive their environment and locate food items in the dark [13], and over 1060 bat species are known to use ultrasound emission to navigate and forage [14]. Apart from species capable of using tongue clicking (*Rousettus* spp.), most bats produce echolocating calls with the larynx, a specialized vocal organ capable of generating high-frequency pulses through rapid oscillation of the vocal membranes [15,16]. Laryngeal calls may be emitted either orally or nasally, producing distinct cranial and head rotations that optimize sound projection [17]. Within nasal emitters, a further morphological difference exists: New World species (Phyllostomidae family) present simple nasal passages, while some Old World nasal emitters have complex nasal chambers. These morphological traits interact with the laryngeal output to shape call frequency, directionality and resonance effects [18–20].

The evolutionary origin of laryngeal echolocation remains a subject of debate. Some studies propose independent acquisitions in different bat lineages (e.g. [21–23]), whereas others suggest a single ancestral origin followed by diversification in call design and cranial morphology (e.g. [24–28]). Call design is a good proxy for preferred hunting habitat as it evolved to meet the environmental challenges associated with different habitat types (i.e. open, edge, clutter habitats [29,30]). Consequently, it is expected to covary with broad cranial morphological variation [31].

Here, we investigated the relationship between cranial morphometrics and echolocation call parameters (i.e. peak frequency, FP) in bats to identify which morphological features covary with FP. Specifically, we tested whether skull morphology, quantified by its size and shape, is adapted to FP in a macroevolutionary context. López *et al*. [32] recently confirmed a functional trade-off between olfaction and echolocation in brain-endocast variation, indicating that cranial morphology is influenced by some echolocation characteristics. We compiled a dataset of 219 species covering almost 65% of laryngeal echolocating bat genera. Species were analysed by emission type as differences between nasal and oral emission represent the main morphological dichotomy in bat skulls associated with echolocation [7, 28, 33, 34]. Other ecological variables (i.e. echolocation call design and diet) were used to identify possible different evolutionary pathways arising from ecological specialization [33].

We applied geometric morphometrics and phylogenetic comparative methods to test the following predictions:

(i) Skull shape and size of non-insectivorous bat species are not constrained by echolocation characteristics (i.e. peak frequency) as they use an integrated sensory system to locate and pursue the prey.
(ii) Variation in call design modulates the link between FP and skull morphology in insectivorous bats, reflecting diverse acoustic constraints.
(iii) FP strongly influences rostrum shape of constant frequency (CF) nasal emitters because of the resonance effect within the nasal chambers.

2. Methods

2.1. Chiropteran sample and deriving functional, ecological and morphological data

We performed evolutionary analyses on 446 specimens belonging to 219 species covering all 19 families of laryngeal echolocating bats. Our dataset covers about 65% of the genus diversity of the order Chiroptera. Specimen details (i.e. museum collections and inventory number) are reported in electronic supplementary material, table S1.

To assess the relationship between morphology and ecological groups, we classified the species by broad diet categories, emission type and call design. Diet was categorized in traditional groups inferred from [35] and is reported in table 1 (see also [9]). Literature was incomplete for species that emit from both the nose and the mouth. Hence, emission type was categorized as oral emission and nasal emission, with the latter divided into New World (i.e. Phyllostomidae species) and Old World (references in electronic supplementary material, table S2) subgroups. Nasal emission implies considerable rearrangements of the skull morphology [34], but different selective pressures might apply to these two groups as nasal chambers in some Old World nasal-emitters are known to behave as resonance structures [18,19].

We used geometric morphometric techniques to collect morphological data on three-dimensional models of bat skulls. The models were reconstructed in three dimensions through either photogrammetry (see protocol in [36]) or micro-CT scans (electronic supplementary material, table S1). The protocol in Giacomini *et al*. [36] demonstrated that multi-technique datasets including models deriving from the use of micro-CT scans and photogrammetry are suitable for assessing the presence and the strength of allometry and other evolutionary parameters in macroevolutionary analyses with a broad phylogenetic scope. A total of 29 landmarks were collected on three-dimensional skull models in order to cover broad anatomical regions and describe the overall cranial morphology. These landmarks (definitions and illustrations in electronic supplementary material, figure S1) are known to reliably capture interspecific cranial shape variation in bats, following previous assessments of measurement error by validation studies [9,36]. Cranial size was extrapolated using the centroid size—the square root of squared distances from each landmark to the centroid of each configuration—which was $\log_{10}$ transformed for subsequent statistical analyses; cranial shape—Procrustes shape coordinates—was obtained from a Generalized Procrustes Analysis on the complete sample of 446 specimens [37]. Both size and shape data were averaged by species for macroevolutionary analyses.

2.2. Allometry and phylogenetic signal

Morphological variation can be misinterpreted unless allometry (i.e. the association between shape and size) and species phylogenetic non-independence are taken into account during macroevolutionary analyses (phylogenetic non-independence [38]; allometry [39]). We tested for the presence of significant phylogenetic signal in morphological traits ($\log_{10}$ centroid size and Procrustes shape coordinates) and in FP using Blomberg *et al*.'s K statistic and its multivariate extension for shape (K_{multiv}) [40,41]. To evaluate the presence and significance of allometry within our dataset while taking phylogenetic relatedness into account, we performed a phylogenetic generalized least squares (PGLS) regression [42,43] with shape (i.e. Procrustes shape coordinates) as the dependent and size (i.e. $\log_{10}$ centroid size) as the independent variable [44].

2.3. Interactions between morphology and eco-evolutionary drivers

Correlations between morphological and functional traits (i.e. categorical variables: diet, emission type, call design; continuous variable: FP) were similarly tested using PGLS regressions. The variance–covariance matrix employed in PGLS analyses [45] was generated from the chiropteran ultrametric and calibrated tree inferred from mitochondrial and nuclear sequence data [46]. Because evolutionary allometry is expected to occur in Chiroptera as whole as well as within taxonomic and ecological subgroups [7], size was included in the regression models as a fixed effect and in interaction with FP when testing for shape variation [47,48]. PGLS analyses were first performed on the complete dataset (n = 219) and subsequently repeated by emission type, call design and family in order to further explore potentially diverse evolutionary patterns due to ecological adaptations.

Table 1. Ecological categories for each group used as independent variables in the present study. Call designs from Jones and Teeling [31].

emission type	call design	diet
Old World nasal emitters	constant frequency (h)	insectivorous
New World nasal emitters	long, broadband, multiharmonic (g)	frugivorous
oral emitters	short, broadband, multiharmonic (f)	hematophagous
	short, broadband, dominated by fundamental harmonic (e)	vertebrate eater
	narrowband, multiharmonic (d)	nectarivorous
	narrowband, dominated by fundamental harmonic (c)	omnivorous
		frugi/insectivorous
		necta/fruigivorous
		insect-vertebrate eater

Shape variation among the 219 sampled bat species was visualized through principal component analysis (PCA) performed on Procrustes shape coordinates of species mean shape. The three-dimensional model of *Cheiromeles torquatus* was the closest to the mean shape (i.e. consensus); therefore, the model was warped on the consensus as reference mesh. This mesh was successively warped on the maximum and minimum shape of the first two PC axes to show major morphological variation in the dataset [49]. The same reference mesh was also employed in the other shape visualizations (i.e. shape deformations related to FP variation). Due to the significant impact of rostral flexion on bat cranial variation [50], we computed the angle between the basicranium (i.e. distance between landmarks 5 and 6) and the palatal plane (i.e. distance between landmarks 6 and 7). We applied a sin transformation to enable PGLS testing of cranial shape components most affected by rostral flexion, improving interpretation of PC axes and allowing comparison of angle values between oral and nasal emitters. We performed all analyses in R software using 'geomorph' [51], 'phytools' [52], 'RRPP' [53] and 'geiger' [54] packages.

3. Results

Morphological variables showed relatively low but significant phylogenetic signal (size: $K = 0.766$, p-value = 0.001; shape: $K_{\text{multiv}} = 0.900$, p-value = 0.001) when compared with FP ($K = 1.306$, p-value = 0.001) supporting the need to apply comparative methods.

3.1. Size and shape variation by ecological groups

Cranial size (i.e. $\log_{10}$-transformed centroid size) variation of the 219 bat species was significantly impacted only by diet categories, which explained approximately 10% of variance, while echolocation type and call designs had no effect (table 2).

Shape variation explained by the first two principal components (PCs) separated species according to mode of echolocation with the oral emitting species scoring lower on PC1 than the nasal emitters (figure 1). PC1 represented variation in relative height and width of the braincase and the length of the palate. Over 30% of PC1 variation was described by the angle between the basicranial and palatal planes (PGLS: $R^2 = 0.326$, p-value = 0.001). Specifically, oral emitters displayed a significantly greater angle between the basicranial and palate planes compared with nasal emitters (PGLS: p-value = 0.010; figure 1). The oral emitters of the genus *Mormoops* showed the greatest angle between the palatal plane and the basicranial one (approx. 231°). PC2 separated species according to their diet category and food hardness, with nectar eaters (i.e. soft food) scoring low and hard fruit eaters scoring high. Shape differences in PC2 were represented by variation in relative cranial height and rostrum length. Species feeding on nectar (e.g. *Choeronycteris mexicana*) displayed long rostra and reduced braincase height. In contrast, hard fruit eaters like the highly specialized *Ametrida centurio*, *Centurio senex* and *Sphaeronycteris toxophyllum*, presented brachycephalic skulls. PGLS analyses confirmed that diet had a significant influence on cranial shape, explaining approximately 7% of the observed variation (similar to the

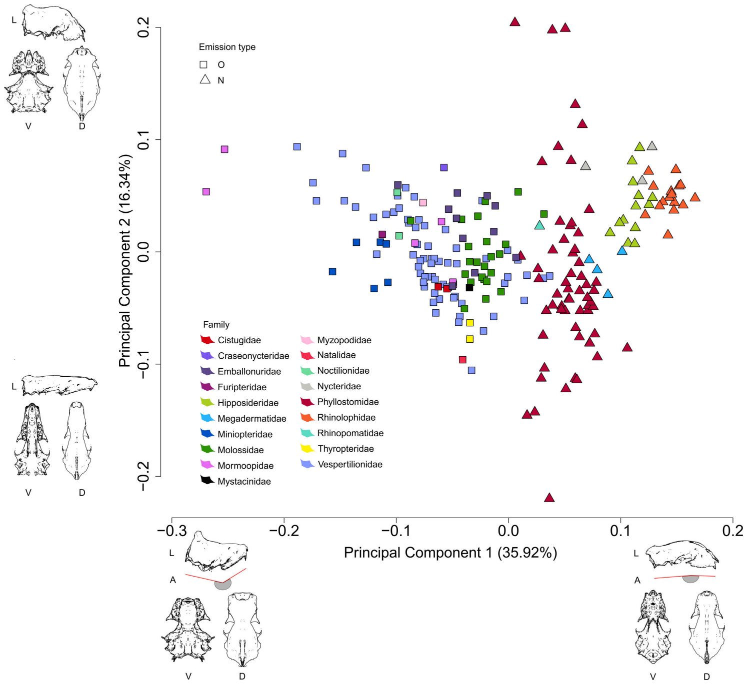

Figure 1. Principal component analysis of 219 species of echolocating bats displayed by family and emission type (O = oral, N = nasal). Shape variation was reported on dorsal (D), ventral (V) and lateral (L) views by warping maximum and minimum PC variation of each axis on *C. torquatus* three-dimensional model. Differences in angles between the basicranium and palate planes (A) were associated with emission type.

Table 2. Results for Procrustes ANOVA models in a phylogenetic framework (PGLS) testing association between three ecological categories and cranial size and shape in a sample of 219 species of bats. Significant *p* values are reported in bold.

	factor	df	R^2	*F*	effect size (*Z*)	*p*
cranial size	emission type	2, 216	0.014	1.481	0.794	0.215
	call design	5, 213	0.044	1.959	1.339	0.085
	diet	8, 210	0.109	3.222	2.757	**0.003**
cranial shape	emission type	2, 216	0.042	4.731	3.064	**0.001**
	call design	5, 213	0.065	2.979	2.259	**0.020**
	diet	8, 210	0.069	1.934	1.913	**0.031**
	size	1, 218	0.067	16.442	6.845	**0.001**
	size × emission type	1, 215	0.016	3.992	3.684	**<0.001**
	size × call design	4, 208	0.035	2.170	2.692	**0.005**
	size × diet	7, 202	0.052	1.851	1.718	**0.048**

proportion accounted for by call design, 6.5% table 2). Echolocation type also had a significant, though weaker, effect on cranial morphology (table 2). Allometric shape variation accounted for roughly 6.7% of total variance, and interactions with the three ecological categories were significant but modest (table 2), consistent with expectations [7,9].

3.2. Size and peak frequency

The impact of FP on skull size variation for all species and within ecological groups (i.e. diet, emission type and call design) under the PGLS model is summarized by figure 2. For the total sample, size strongly correlated with FP (PGLS: R^2 = 0.214, p-value = 0.001). Specifically, species with bigger heads presented lower FP (PGLS β coefficient = −0.287). Skull size of insectivorous bats presented the highest correlation with FP (PGLS: n = 161, R^2 = 0.307, p-value = 0.001), while frugivorous species and other bats did not show allometric effects for FP (PGLS: n = 21, R^2 = 0.051, p-value = 0.317; n = 37, R^2 = 0.053, p-value = 0.176, respectively).

Within the insectivorous bats, we repeated the test separately by emission type. Oral emitters presented a slightly weaker correlation compared with nasal emitters (PGLS: n = 119, R^2 = 0.338, p-value = 0.001; n = 42, R^2 = 0.352, p-value = 0.001, respectively). Within the nasal emitters, some species shifted from the allometric pattern. Specifically, *Rhinopoma microphyllum* and *Macrophyllum macrophyllum* were smaller in head size than predicted by their FP while *Hipposideros diadema* was larger than expected (figure 3a). Furthermore, Old-World nasal emitters (i.e. Rhinolophidae, Megadermatidae, Nycteridae, Rhinopomatidae) showed the strongest allometric relationship (PGLS: n = 32, R^2 = 0.497, p-value = 0.001; electronic supplementary material, figure S2).

Within the oral emitters, two of the most diverse families (Vespertilionidae and Molossidae) showed different allometric effects on FP (figure 3b). The lowest impact of size on FP was found in the Vespertilionidae (PGLS: n = 64, R^2 = 0.224, p-value = 0.001), while the greatest impact was found in the Molossidae (PGLS: n = 23, R^2 = 0.520, p-value = 0.001). Only *Cheiromeles torquatus* showed deviations from the association between size and FP within the Molossidae family (electronic supplementary material, figure S3). The Vespertilionidae showed considerable variance around the estimated allometric relationship, therefore supporting the lower R^2 relative to the Molossidae (electronic supplementary material, figure S4).

Species emitting different types of calls displayed different strengths of association between FP and skull size (figure 2b). Bats emitting components of CF sounds (‘h’: i.e. Rhinolophidae, Hipposideridae and *Pteronotus parnellii*) showed the highest allometric effect (PGLS: n = 30, R^2 = 0.586, p-value = 0.001; electronic supplementary material, figure S5). Skull size of narrowband multiharmonic (d) and monoharmonic (c) emitting species showed a lower but still strong correlation with FP (PGLS: n = 20, R^2 = 0.350, p-value = 0.006; n = 65, R^2 = 0.421, p-value = 0.001, respectively; electronic supplementary material, figures S6 and S7). Species emitting broadband with fundamental harmonic only (e) presented the wicker allometric effect (PGLS: n = 25, R^2 = 0.191, p-value = 0.024; electronic supplementary material, figure S8). Only the skull size of bats emitting broadband multiharmonic signals (f) did not show an allometric effect for FP (PGLS: n = 20, R^2 = 0.002, p-value = 0.867). There was only one sampled species emitting call type ‘g’ (i.e. *Myzopoda aurita*) so no statistical test was applied to this category.

3.3. Shape and peak frequency

Associations between skull shape and FP for all samples and within ecological groups are summarized in figure 2. The shape of 219 species of bats significantly correlated with FP after phylogenetic correction (PGLS: R^2 = 0.015, p-value = 0.002) when accounting for size. Species with higher peak frequencies presented narrower rostra with shorter maxillae (i.e. shorter nasal chamber area) and a decreased relative size of the tympanic bullae for higher frequencies (figure 4a). When this association was explored by diet, skull shape in frugivorous bats presented the highest correlation with FP (PGLS: R^2 = 0.154, p-value = 0.001) while insectivorous species followed the overall pattern described above (PGLS: R^2 = 0.017, p-value = 0.002). Other bats did not present a significant association between skull shape and FP (PGLS: n = 37, R^2 = 0.028, p-value = 0.336). Frugivorous species emitting high peak frequencies presented a shorter and narrower maxilla with increased cranial vault depth. The palate was shorter but wider, and the relative size of the tympanic bullae decreased for higher frequencies (figure 5). The highly specialized hard-fruit eaters, i.e. *Ametrida centurio*, *Centurio senex* and *Sphaeronycteris toxophyllum*, showed the same pattern but with a steeper slope compared with the other fruit eaters.

Within the insectivorous bats, we repeated the test by emission type, identifying two general patterns of association between skull shape and FP dependent on emission strategy (i.e. nasal and oral). As for size, oral emitters presented a weaker correlation between shape and FP compared with nasal emitters (PGLS oral: n = 119, R^2 = 0.018, p-value = 0.019; PGLS nasal: n = 42, R^2 = 0.061, p-value = 0.002, respectively). In both groups of nasal emitters (i.e. New and Old World), high frequencies were associated with narrower and shorter nasal chambers (figure 4a). High frequencies were associated with narrow palates in oral emitters too, but the overall skull was more elongated (palate included, figure 4a). The relative size of the tympanic bullae decreased at higher frequencies in

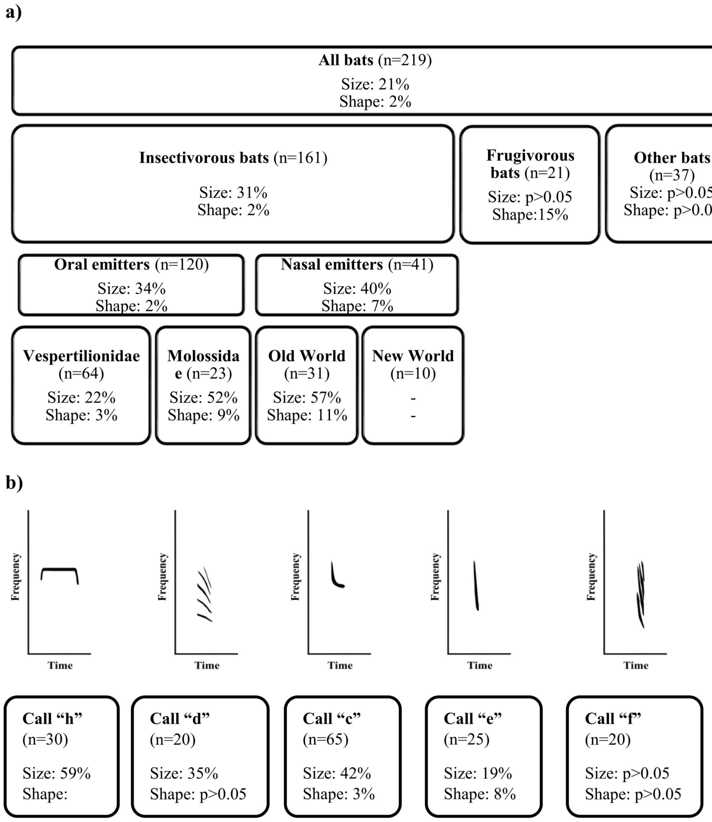

Figure 2. Size and shape correlation with FP by emission type (a) and by call design for insectivorous bats (b). Spectrograms of call designs not in scale. Call 'h': Rhinolophidae ($n = 16$), Hipposideridae ($n = 13$), *P. parnellii*; call 'd': Emballonuridae ($n = 11$), Mormoopidae ($n = 5$), Thyropteridae ($n = 2$), *Craseonycteris thonglongyai* and *Rhinopoma microphyllum*; call 'c': Cistugidae ($n = 2$), Miniopteridae ($n = 6$), Molossidae ($n = 23$), Vespertilionidae ($n = 34$); call 'e': Vespertilionidae ($n = 24$) and *Furipterus horrens*; call 'f': Phyllostomidae ($n = 10$), Vespertilionidae ($n = 6$), *Megaderma spasma, Mystacina tuberculata, Natalus tumidirostris* and *Nycteris hispida.*

both nasal and oral emitters. Some species deviated from the nasal-emitter pattern, specifically *Nycteris hispida* and *Lonchorhina aurita*, which presented a longer rostrum and hard palate than predicted by their FP, and *H. diadema*, presenting a shorter premaxilla and palate than expected (figure 4b). In the Old-World nasal emitters, FP explained almost 10% of skull shape variance under the PGLS model ($n = 32$, $R^2 = 0.094$, p-value = 0.001; electronic supplementary material, figure S9).

As for the size of mouth emitters, the relationship between shape and FP varied considerably within families. Moreover, *Mormoops* species, *Furipterus horrens* and *Craseonycteris thonglongyai* deviated from the overall pattern of oral emitters (figure 4c). Species from the Molossidae family presented a higher correlation between shape variables and FP (PGLS: $n = 23$, $R^2 = 0.087$, p-value = 0.011) compared with the Vespertilionidae (PGLS: $n = 66$, $R^2 = 0.033$, p-value = 0.021). Molossidae species displayed a shorter but wider rostrum and a longer braincase at higher frequencies (electronic supplementary material, figure S10). In accordance with the deformation pattern of the oral emitters, Vespertilionidae presented longer braincases and shorter rostra (but slightly longer palates) and smaller tympanic bullae (electronic supplementary material, figure S11). *Lasiurus cinereus*, *Glischropus tylopus*, *Chalinolobus gouldii* and *Vespertilio murinus* showed wider and shorter rostra than expected by their FP. On the contrary, some of the big *Myotis* species (*M. myotis*, *M. blythii* and *M. welwitschii*) and the medium-sized *M. simus* showed narrower and longer rostra, and shorter braincases than expected.

Insectivorous species emitting echolocation calls with different structures showed differences in the pattern of association between skull shape and FP (figure 2b). Specifically, nasal-emitting bats producing CF calls presented the highest correlation between shape and FP (PGLS: $n = 30$, $R^2 = 0.115$, p-value = 0.001; electronic supplementary material, figure S12). Species emitting 'c' signals showed a weaker but still significant correlation between shape and FP (PGLS: $n = 65$, $R^2 = 0.030$, p-value = 0.049; electronic supplementary material, figure S13). Species relying on broadband monoharmonic calls (e) showed a significant relationship between shape and FP (PGLS: $n = 25$, $R^2 = 0.071$, p-value = 0.033; electronic supplementary material, figure S14). Finally, species emitting broadband and narrowband

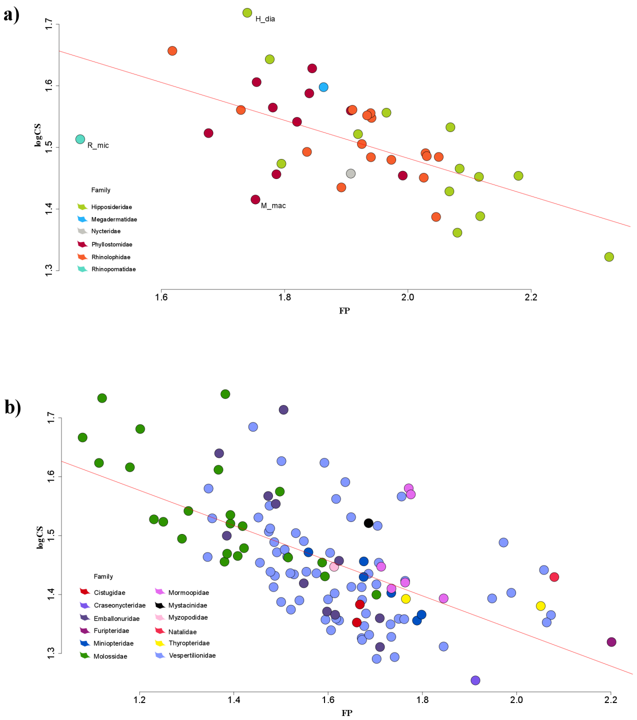

Figure 3. Allometric effect on FP for insectivorous bats: nasal emitting species (a) and oral emitting species (b). Colours represent families and labels design the outliers (H_dia: *Hipposideros diadma,* M_mac: *Macrophyllum macrophyllum,* R_micr: *Rhinopoma microphyllum).*

multiharmonic signals (i.e. ‘f’ and ‘d’ calls) did not show a correlation between skull shape and FP (PGLS: $n = 20$, $R^2 = 0.076$, p-value = 0.114; $n = 20$, $R^2 = 0.035$, p-value = 0.770, respectively).

4. Discussion

In this study, we found that the skull shape of insectivorous and frugivorous species presents adaptations to specific echolocation frequencies. Functional demands linked to echolocation seem to strongly influence skull shape, also of those species integrating multiple sensory systems to locate the food (i.e. frugivorous species), contrary to our first prediction. Relative rostrum length/width and tympanic bulla size were found to covary with frequencies emitted across all taxa and most ecological groups consistently. Different emission types and call designs imply different evolutionary constraints on bat skull morphology. This was evident in constant-frequency (CF, call type ‘h’) species, which consistently showed strong associations between FP and both cranial size and shape, supporting our second and third predictions. Significant FP effects were also observed in species emitting monoharmonic (c) and broadband fundamental (e) calls. It is of note that monoharmonic species deviated from the general deformation pattern by showing no relationship between relative rostrum length and FP. Based on these findings, two non-exclusive mechanisms may be formulated to explain cranial morphological changes relative to FP: (i) a physical acoustic principle, such as resonance effect or harmonic filtering, drives the direct co-evolution between shape and frequency emitted (physical acoustic hypothesis); (ii) FP directly coevolved with echolocating muscles; therefore, the spatial and mechanical demands of the muscles moulded the shape of the skull (mechanical hypothesis).

4.1. Palate orientation and head position

Oral emitters were found to have wide palatal–basicranial angles (i.e. palate elevated with respect to the basicranium), suggesting that an upward-tilted skull might promote effective sound projection through the mouth. The projection of the sound is also probably facilitated by the upward position of the head during the flight by oral emitters [55]. We suggest that this configuration imposes different constraints on the head muscles and bones of oral emitters with respect to nasal emitters and may

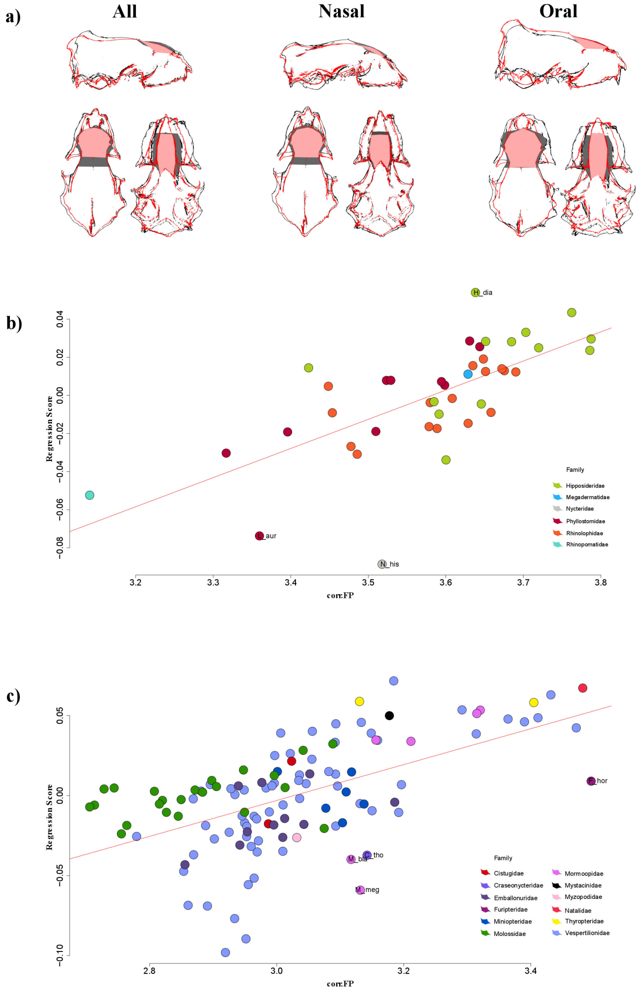

Figure 4. Shape deformations (a) for all insectivorous bats, nasal emitting species and oral emitters: the black and red outlines describe the species with lowest and highest FP, respectively. Hard palate and rostrum are highlighted in grey and pink, respectively. Plot of shape (as regression score) and size-corrected FP for insectivorous bats: nasal emitting species (b) and oral emitting species (c). Colours represent families and labels design the outliers (nasal emitters: H_dia: *Hipposideros diadma,* N_his: *Nycteris hispida,* L_aur: *Lonchorhina aurita;* oral emitters: C_tho: *Craseonycteris thonglongyai,* F_hor: *Furipterus horrens,* M_meg: *Mormoops megalophylla,* M_bla: *Mormoops blainvillei*).

explain the nature of the relationship between skull shape and FP in oral emitting bats. Ontogenetic studies revealed that the orofacial complex of nasal-emitting bats goes through different developmental stages compared with other mammals [17]. In oral emitters, the orofacial complex rotates dorsally on the basicranium when the head unfolds from the chest during pre-natal growth, similarly to other non-echolocating mammals. Conversely, there is no dorsal rotation of the palate in nasal-emitting species, and this anatomical configuration optimizes the alignment of the nasal passage with the larynx [34]. Therefore, the combined effects of head rotation, palate orientation and flight posture likely contribute to efficient sound projection through the mouth or nose in echolocating species (figure 6).

4.2. Size and peak frequency

Peak frequency scales with body size in insectivorous bat species [11]. Insectivores with small bodies produce high frequencies because of a physical acoustic principle, i.e. short/thinner acoustic folds and smaller resonance structures. Furthermore, a small body size in a flying animal increases manoeuvrability and, as a consequence, likely improves hunting success in a cluttered environment [56]. High-frequency sounds are advantageous in a cluttered environment as they reduce scatter echoes from the background [30]. Therefore, ecology and physical acoustics regulate the relationship between FP and skull size. In our study, even when non-insectivorous species were excluded from the sample,

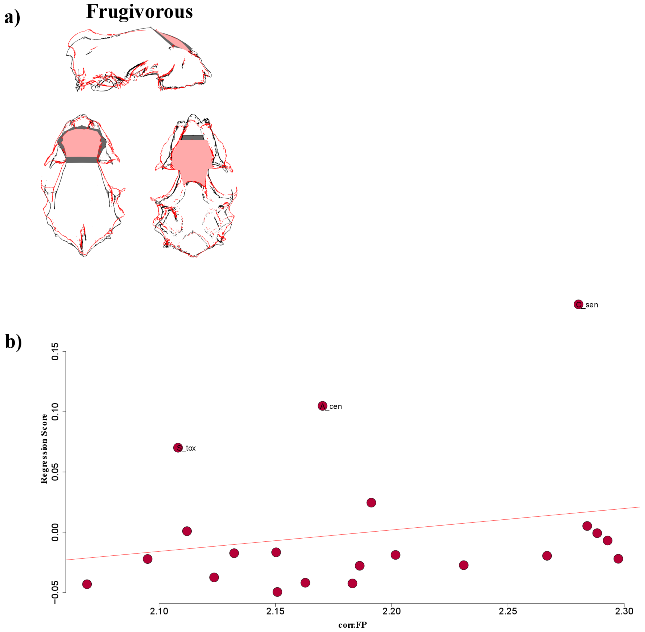

Figure 5. Shape deformations (a) for frugivorous bats ($n = 21$) belonging to the Phyllostomidae family. The black and red outlines describe the species with lowest and highest FP, respectively. Hard palate and rostrum are highlighted in grey and pink, respectively. Plot of shape (as regression score) and size-corrected FP for frugivorous bats (b). Labels designate the outliers (A_cen: *Ametrida centurio*, C_sen: *Centurio senex*, S_tox: *Sphaeronycteris toxophyllum*).

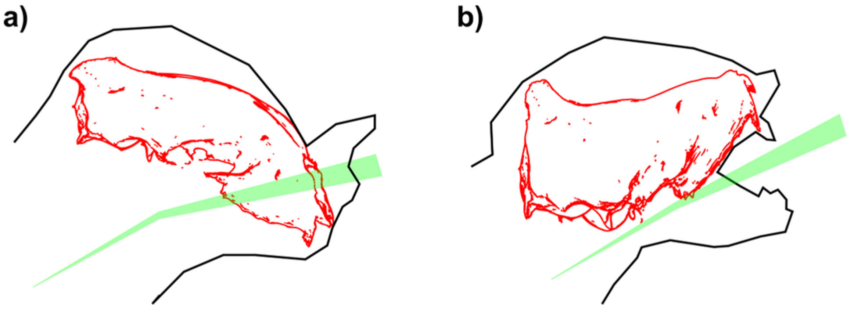

Figure 6. Head axis rotation [34] and positioning during echolocation [55] in nasal emitting species (a) and oral emitting species (b). In oral emitters, the basicranium–palatal plane resulted 'tilted'.

some species still deviated from the allometric pattern of their ecological category. Deviations from the allometric relationship can be explained by different non-mutually exclusive hypotheses [57]. Species able to deviate from the pattern either (i) exhibit specialized hunting strategies where larger skulls (and hence heavier bodies) are not disadvantageous, particularly for behaviours such as gleaning and perch-hunting; (ii) their frequency range is modified according to prey size (valid only for bats emitting low frequencies; e.g. [58]); (iii) their sound emission diverges from the acoustic detectability range of eared moths in order to increase their hunting success; and (iv) their frequencies present acoustic displacement to facilitate intraspecific communication.

Different slopes in the allometric pattern were identified among different families of oral emitters, indicating the impact of phylogenetic relationships on the strength of size-shape covariation. These results complement recent work demonstrating that sound emission modes shape allometric relationships and modularity in echolocating bats, contributing to multiple adaptive zones in cranial evolution [7,33]. Molossidae presented the strongest allometric effect, with only the greater naked bat (*C. torquatus*) deviating from the pattern of a larger skull size, which is predicted by FP. It has been proposed that morphological divergence in Molossidae is related to dietary specialization, specifically to prey hardness [59]. *Cheiromeles torquatus* is the largest aerial hawking insectivorous bat (approx. 160 g), with a skull morphology that predicts it to be a hard food species [60]. It is considered to be a fast-flying species [61]. Hence, the detection of small insects might be limited by a low-frequency call (approx. 24 KHz) as the wavelength might not be long enough to produce informative echoes [62].

Therefore, *C. torquatus* might have evolved a higher frequency to detect prey otherwise not detectable at the predicted frequency of 7 KHz for its body size [60].

Phyllostomidae did not show a relationship between skull size and FP (see additional results in electronic supplementary material) in accordance with previous studies [11]. The echolocation call structure of most phyllostomid bats suggests that they are gleaners [63] and use additional sensory cues to locate their food (e.g. vision, olfaction and prey-generated acoustic cues [64,65]). However, some flexibility may be present in the strategy of these species (e.g. *M. macrophyllum*, see above). It has been theorized that nose-leaf size might scale with FP instead of skull size [11]. This is particularly plausible for nasal emitting species, considering that sound diffracts from the nostrils and its acoustic properties (e.g. directionality) are influenced by the geometry of the channels and the 'baffle' (i.e. nose-leaf) [66,67].

Adaptation of skull size to FP was stronger for species producing CF calls (call type 'h') and call type 'c' (in particular from Molossidae family). These two groups of echolocators use the extreme range of frequencies: high frequencies for CF species and low frequencies for Molossidae bats. All species producing call type 'h' are nasal emitters, except for *P. parnellii*. These species experience a resonance effect when the sound travels inside their nasal chambers; therefore, size adjustments are fundamental to 'tune' the cavity and enhance the correct frequency [18,19]. The resonance effect is not relevant for mouth emitters and therefore, in Molossidae species, FP possibly coevolved with size to increase niche partitioning between ecologically similar species.

Insectivorous species emitting call type 'e' (i.e. Vespertilionidae and *Furipterus horrens*) showed the weakest correlation between size and FP. These species emit in the medium–high frequency range (from approx. 32 KHz of *Scotomanes ornatus* to approx. 160 KHz of *F. horrens*, in our sample) and they display different hunting strategies [30]. It is worth mentioning that FM calls are characterized by a long sweep of frequencies and the energy of the call is more equally distributed along this sweep than in other call types. Therefore, the size of the echolocator system (i.e. skull and echolocating muscles) might be less influenced by one specific frequency (FP) within this group. Conversely, in these species, we found a stronger relationship between FP and shape (see next section). Concerning the entire order of Chiroptera, the results from our analyses, which rely on size estimations deriving from geometric morphometrics, are in line with recent research (relying only on literature-based body masses) suggesting a strong impact of echolocation and call duration on chiropteran body size (68).

4.3. Shape and peak frequency

The relative strength of association between shape and echolocation followed a similar pattern to the correlation between size and FP, with two exceptions: frugivorous and call type 'e' insectivorous species. Our results confirmed that skull shape of insectivorous species is influenced by FP in a macroevolutionary sample with high taxonomic coverage (n = 161). A shorter rostrum (i.e. maxilla) was associated with high frequency in all ecological groups. Species emitting broadband, monoharmonic echolocation calls (i.e. call 'e') were found to be the exceptions to this rule. In these cases, FP variation was not associated with relative rostrum length but rather rostrum width. Furthermore, in our study, the tympanic bulla were proportionally larger for species emitting lower FP across all taxa, when allometry was accounted for. Large tympanic cavities are believed to be an adaptation towards improving low-frequency hearing in terrestrial mammals [69]. In bats, nearly all middle ear components—tympanic membrane, pars flaccida and stapes—are smaller in species that emit high frequencies [70]. Proportionally smaller auditory bullae in these species may indicate additional adaptations for enhanced sensitivity within specific frequency ranges.

The adaptation of bat skull shape to emitted frequencies can result from a direct pressure by the physical constraints underlying hearing or can be the indirect outcome of selective forces exerted by echolocating muscles. Echolocation (and diet) was shown to have a role in brain-endocast correspondence of the olfactory bulbs, indicating a trade-off between olfaction and echolocation [32]. The short rostrum present in all nasal emitters (New and Old World) indicates that resonance effects might have a direct impact on the nasal chamber shape. Therefore, acoustic dynamics might explain the adaptation of nasal chamber shape to FP in nasal emitting species (particularly call 'h' species). However, indirect skull shape adaptation to muscles specialized for echolocation, and thus to FP, might better explain the results for mouth emitters.

Within the oral emitters, the relationship between skull shape and echolocation appears to be driven by the muscles used for echolocation (phonetic system) causing complex patterns of adaptation between the sensory system and morphology. The position and size of the laryngeal muscles might have strong consequences on the shape of the skull. Plotsky *et al*. [71] showed that larynx repositioning is associated with craniofacial variation in dogs. Furthermore, the anatomy of the larynx and hyoid apparatus is also known to be different for bats producing CF calls (call type 'h') compared with those producing frequency-modulated calls (call type 'e' [72]). Insectivorous bats evolved big and fast laryngeal muscles, in particular cricothyroid muscle, to control the tension and oscillation of the vocal folds during the production of ultrasound [73]. It is possible that variation in the larynx muscles, which are under direct evolutionary pressure due to echolocation, drives variation in skull shape.

Our results suggest that the functional demand of echolocation in nasal emitting species might be stronger compared with oral emitters. As predicted, call type 'h' emitting species showed the highest association between FP and shape, indicating that relative size of the rostrum (therefore nasal chamber) is adapted to further increase the resonance effect. As for size, the skull shape of Molossidae bats (all 'c' type call emitters) showed the highest association between skull shape and echolocation within the oral emitters. All Molossidae species emit a mixture of frequency-modulated and quasi-CF calls and with the exception of *Molossops temminckii*, they are all aerial hawking hunters in open space [63]. There are some similarities with the Rhinolophidae, where all species present a similar diet, call design (call type 'h') and hunting strategy (narrow space flutter detecting forager) [30]. Therefore, the relationship between FP and skull shape may be easier to detect in these families compared to Vespertilionidae where different hunting strategies evolved (therefore, the relationship between shape and FP potentially has different slopes relative to hunting strategy). This is supported by the fact that echolocation parameters correlate with wing morphology in Vespertilionidae [74] but not in Rhinolophidae [75].

Contrary to our expectations, the skull shape of monoharmonic FM emitting species (some Vespertilionidae and *F. horrens*) appears co-adapted to FP. This is particularly surprising given that FM calls are characterized by a long sweep of frequency that makes parameterization challenging and potentially less stable. Within this pattern, *Myotis* species showed a different skull shape for similar emitted frequencies, suggesting that FP might not be coadapted to skull shape in these species. Different hunting strategies have evolved in this genus in order to avoid food competition (e.g. [76,77]), and therefore, environmental and prey specialization might exert a stronger evolutionary pressure on FP than skull morphology in *Myotis* spp.

Our hypothesis that non-insectivorous bats would not present a correlation between skull shape and echolocation has been disproven for frugivorous species. In the Phyllostomidae family, we found that cranial shape, in particular rostrum relative size and braincase height, has co-evolved with echolocation parameters. This pattern seems even stronger for hard fruit eaters, suggesting that also highly specialized species are constrained by functional demands related to echolocation. At least two of the three hard fruit eaters produce monoharmonic broadband calls (call 'e') different from the multiharmonic calls of other fruit eaters (call 'f'). Similar to blood, nectar and vertebrate eaters, most frugivorous bats rely on both active echolocation and other sensory strategies, but it is still unclear to what extent the shift between sensory strategies is flexible and if there are species that rely on one sensory system only. Even if a trade-off between vision and echolocation has been hypothesized for Phyllostomid bats [78], there is currently no evidence of nasal chamber morphological adaptation to olfaction [79]. Whether the morphological adaptation of the nasal passages to echolocation is stronger, or simply more evolutionarily resilient, than olfactory ones still needs to be investigated. Both insectivorous and frugivorous nasal emitters (including call 'h' emitters) presented short rostra at high frequencies, suggesting that decreasing relative volume of nasal passages is an adaptation to high frequency emission regardless of the diet or phylogenetic history. Nevertheless, it is unlikely that skull adaptations to FP in frugivorous species allow for the same magnitude resonance effect as in call 'h' emitting species. Most Phyllostomidae species, indeed, shift energy between different harmonics of the broadband call (call 'f'; e.g. [80]) challenging the acoustic tuning of the nasal passages. The 'mechanical hypothesis' could be extended and used to explain how the association between FP and skull shape in frugivorous species arises from FP adapting to nose-leaf form—an acoustic baffle—and to the maxillary structures that support it.

5. Conclusions

Our results improve the understanding of the factors influencing bat skull evolution by highlighting that a relevant portion of size variation is explained by shared evolutionary history, FP and diet. Evolutionary pressures on shape variation occurring within chiropterans appear to be persistent and preserved, with phylogenetic relatedness only partially driving variation in skull shape. Diet specialization and emission type are drivers of shape differentiation at the order level, whereas echolocation parameters play a role at a lower ecological (i.e. within single categories) and taxonomic (i.e. within single families) scales. A great proportion of bat skull shape variation remains unexplained. Even if call design represents a proxy for hunting strategy, more detailed analyses of foraging guilds and habitat complexity might further clarify the coevolutionary pattern between skull morphology and echolocation within some bat families (e.g. Vespertilionidae).

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Original three-dimensional landmarks that compose this dataset are available at https://figshare.com/s/b41e112365c076f7b325.

Supplementary material is available online [81].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. G.G.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, software, validation, visualization, writing—original draft, writing—review and editing; D.T.: formal analysis, investigation, methodology, software, validation, visualization, writing—review and editing; A.H.: conceptualization, data curation, methodology, resources, supervision, validation, writing—review and editing; R.B.: conceptualization, investigation, methodology, project administration, supervision, validation, writing—review and editing; R.P.-M.: data curation, resources, supervision, validation, writing—review and editing; C.M.: conceptualization, investigation, methodology, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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