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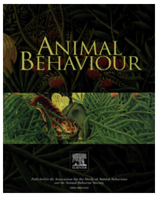

Male forehead gland scent may encode multiple information in the great Himalayan leaf-nosed bat, *Hipposideros armiger*

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Intrasexual competition favours the evolution of pronounced individual identity and competitive ability badges. However, although olfactory signals play a role in territorial conflicts of many animals, the information content encoded in olfactory signals in the context of territorial conflicts is relatively unknown. Here, we used male great Himalayan leaf-nosed bats, *Hipposideros armiger*, to explore whether olfactory signals from the forehead gland encoded reliable information about a signaller's individual identity, physical condition, dominance status and physiological quality. We did this by using gas chromatography—mass spectrometry to quantify the chemical composition of the gland exudate and by examining the relationships between the proportions of 24 volatile compounds and physical condition (body condition index), dominance status (David's score) and physiological quality (immunocompetence, hormonal levels and resting metabolic rate). The results showed that there were significant differences in the concentrations and categories of compounds between individuals. Moreover, the proportions of many compounds correlated with David's score and neutrophil/lymphocyte ratio. Our results indicate that olfactory signals of *H. armiger* could transmit information about individual identity, dominance rank and physiological quality and may work as a reliable 'chemical ornament' for male—male competition.

Fighting for limited resources, such as food, mates or territories, is commonplace in the animal kingdom (Bradbury & Vehrencamp, 2011). Costly conflicts can lead to energy expenditure, physical injuries and even death. However, conflict resolution without resorting to violence is common and often results when the opponents exchange information via agonistic signals to determine the superior individual before escalating to a physical fight (Briffa, 2015). Like acoustic signals, olfactory signals are vital agonistic signals and may provide reliable information to opponents about the resource-holding potential of the signallers and thus permit competitors to assess a rival's relative competitive ability and avoid costly physical contests (Bradbury & Vehrencamp, 2011).

Olfactory signals containing information about individual identity have been documented in various taxa, including insects (Lenoir et al., 1999), amphibians (Chouinard et al., 2013), reptiles (Moreira et al., 2006), birds (Mardon et al., 2010) and mammals (Burgener et al., 2009). Individual recognition via olfactory signals can play a crucial role in territorial defence (Wyatt, 2014), and the efficient discrimination of competitors could decrease territorial conflicts with competitors that have a high fighting ability (Gosling & McKay, 1990).

Olfactory signals can also encode information about body condition (López et al., 2006). For example, the body condition index of male Carpetan rock lizards, *Iberolacerta cyreni*, was positively associated with the proportions of oleic acid secreted by the femoral gland (Martín & López, 2010). As with individual identity signals, the relationship between olfactory characteristics and body condition may result from high costs of signal production or maintenance, making it difficult for low-quality senders to produce a signal (López et al., 2006).

Olfactory signals can also convey information about dominance rank in many species, such as lizards (Moreira et al., 2006), mice (Macholán et al., 2023) and monkeys (Setchell et al., 2010). Specifically, selective pressures may drive social animals to clearly signal their dominance states to competitors when they live in

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groups with a linear dominance hierarchy. This information may facilitate stable social relationships (Pusey & Packer, 1997). For instance, spotted hyaenas, *Crocuta crocuta*, have a linear dominance hierarchy, and there is a significant difference in the composition of scent profiles in anal glands between high-ranking and low-ranking individuals (Burgener et al., 2009).

In addition to individual identity, body condition and dominance rank, olfactory signals can provide information about individual physiological quality such as hormonal levels (Alberts et al., 1992) and immunocompetence (Rantala et al., 2002). For instance, in green iguanas, *Iguana iguana*, the percentage of lipids in femoral gland secretions is positively associated with plasma testosterone (T) levels, indicating that olfactory signals transmitted in the lipids may provide information about hormonal levels (Alberts et al., 1992). In male Iberian rock lizards, *Lacerta monticola*, individuals with a greater T-cell immune response have higher proportions of two steroids in their femoral gland secretions (López et al., 2006).

Bats not only rely on acoustic signals (e.g. echolocation calls and social calls) for navigation, prey detection or communication but also commonly use complex olfactory signals for social activities in the dark habitats they often occupy (Gillam & Fenton, 2016). Except for mice, olfactory communication is generally not well understood in mammals, and this is particularly true of bats. Moreover, our knowledge of olfactory communication in bats lags behind our understanding of acoustic communication (Chaverri et al., 2018). Nonetheless, different integumentary glands and nonglandular odour-producing structures have been described from different regions of the bats' body (Muñoz-Romo et al., 2021). Previous studies on information encoded in olfactory signals of bats have largely focused on the context of courtship or mating (Adams et al., 2018; Caspers et al., 2008; Faulkes et al., 2019); whether olfactory signals of bats can convey different types of information in the context of territorial conflicts is not well studied.

Here, we examine whether olfactory signals encode honest information about individual attributes of male great Himalayan leaf-nosed bat, *Hipposideros armiger*, during agonistic interactions. *Hipposideros armiger* is a nocturnal and highly social species with a polygynous mating system (Yang, 2011). Hundreds of individuals roost in caves where they maintain a minimum distance of 10–15 cm from each other during daytime roosting (Sun et al., 2019). They defend their day-roost territory using multimodal displays incorporating visually conspicuous agonistic displays, territorial calls and forehead gland odours (Sun et al., 2018; Zhang et al., 2022; Fig. 1a). Understanding the relationship between olfactory signals and individual attributes is crucial for the interpretation of our previous results that the olfactory signals from forehead gland secretions of male *H. armiger* serve the function of territorial defence during the agonistic interactions (Zhang et al., 2022). We hypothesized that olfactory signals emitted by male *H. armiger* would predict (1) their individual identity, (2) physical condition, (3) dominance status and (4) physiological quality (hormone levels, immunocompetence and resting metabolic rates). As a test of these hypotheses, we predicted that (1) male *H. armiger* olfactory signals would encode detectable individual-specific scent profiles, and olfactory parameters would correlate with (2) body condition index, (3) individual dominance score and (4) physiological parameters including corticosterone (CORT) and T levels, immunocompetence-related parameters and resting metabolic rate. Note that prediction (3) about dominance status assumes that dominance status is linear and relatively stable because otherwise potential information encoded in an olfactory signal would not be robust. Therefore, in addition to testing our four predictions, we also tested for the linearity of the dominance hierarchy.

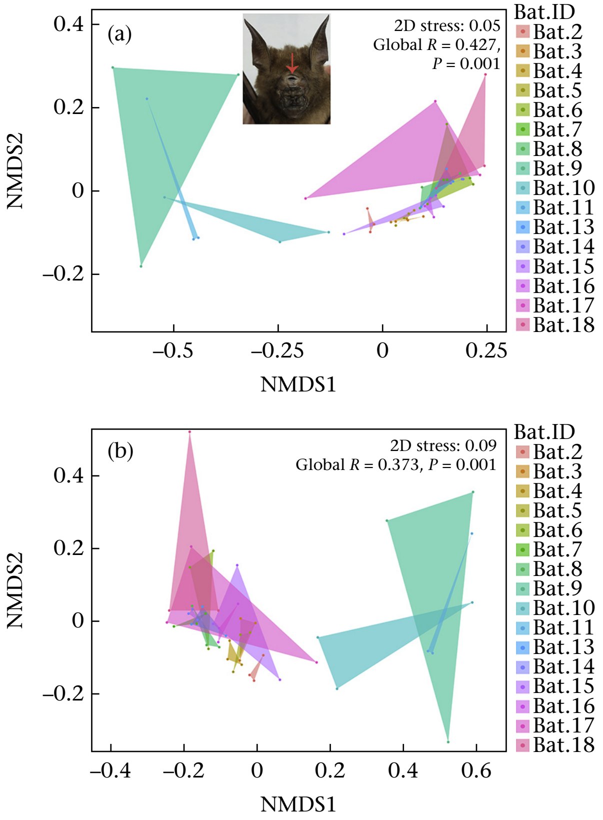

Figure 1. Nonmetric multidimensional scaling plots showing (a) the similarity in chemical composition of 48 samples from 16 individuals and (b) chemical similarity of major categories of compounds identified from the forehead gland of 16 bats. The red arrow in the inserted picture of a great Himalayan leaf-nosed bat indicates the location of the forehead gland.

METHODS

Ethical Note

Our work adheres to the ASAB/ABS Guidelines for the Use of Animals in Research and to the National Natural Science Foundation of China for experiments involving vertebrate animals and was approved by the Animal Research Ethics Board of Hebei Normal University China (approval number: HEBTU-2022LLSC044). No bat suffered any obvious injuries from capture and transport, and our experiments did not result in any physical injuries or death. Bats were always handled gently. No visible physical injuries were observed, although some contests involved physical contact. All bats were released in good health at their original site of capture after the completion of the experiments. The study was conducted during the nonbreeding season (from November 2021 to March 2022).

Animals and Housing

Eighteen adult male *H. armiger* were captured with a mist net from the Baishui cave in Jiangkou County, Tongren City, Guizhou Province, China (108.83°E, 27.68°N), on 20 November 2021. The assemblage of *H. armiger* from the Baishui cave was considered to be a single colony. Captured bats were transported by an air-conditioned vehicle from the field to the laboratory at Hebei Normal University, China. They were housed together in a climatic chamber (length × width × height: 2.1 × 1.8 × 2.2 m) at a relative humidity around 70%, a temperature at around 22 °C, and a 12:12 h light:dark cycle (dark: 1900−0700 hours; light: 0700−1900 hours). Based on our extensive observations in the lab (C. Sun, personal observation), all bats could engage in free flight in the climatic chamber. Moreover, none of the bats got their claws stuck in the wire mesh with 1 × 1 cm mesh. The space in the lab did not affect the positioning of the individuals or their agonistic interactions in captivity because the spatial arrangement of bats in the experimental space was similar to the spatial arrangement under natural conditions (C. Sun, personal observation). For individual identification, we marked all bats using aluminium alloy bands. All bats were fed *Zophobas morio* larvae and given freshwater enriched with vitamin and mineral supplements ad libitum. All bats stayed in the climatic chamber for 127 days (Table 1).

Tagging Procedures

When the captured bats were transferred to housing in the lab on 20 November 2021, we marked the bats with numbered aluminium alloy bands (2.9 mm for the leg; 4.2 mm for the forearm; Porzana, East Sussex, U.K.) on either the leg, the forearm or a combination of leg and forearm. Based on our recent study (Sun et al., 2021), we ensured that the bands could slide loosely on the forearm and the leg and did not affect the normal behaviour of the experiment individuals. Detailed band positions for each bat can be found in Table S1 and in Figs. S1 and S2. After the dominance hierarchy determination, to reduce the potential injury of the bands to bats, we removed the extra bands and only kept one band on the forearm for each bat (Table S2 and Fig. S3). In addition, given that two of the 18 bats (bat.1 and bat.12) were excluded from subsequent analyses due to inadequate secretion for the sampling periods, we removed the bands from those two bats (Table S2 and Fig. S3). We found that of the 16 bats that were tagged, two (bat.2 and bat.8; 12.5%) showed mild skin lesions derived from the right forearm ring, and no skin lesions were detected in the remaining 14 bats (78.5%). We disinfected the forearm wound with iodophor every other day until recovery. After the resting metabolic rate monitoring, we removed all 16 bats' bands and kept them in the climatic chamber for a resting period before releasing them (Fig. S4). No skin lesions were detected in any of the 16 bats at this stage. After a 30-day resting period, all 18 bats were released in good health at their original site of capture (Video S1). A detailed experimental schedule can be found in Table 1.

Physical Condition Measurement

We used body condition index (BCI) as a proxy for the bats' physical condition. BCI was calculated as the body mass divided by forearm length. We measured forearm length of each individual to the nearest 0.01 mm using an electronic Vernier calliper (111-101V-10G, Guanglu, Shenzhen, China) and measured body mass of each male using an electronic balance (±0.01 g; DH-I2000, Diheng, Shenzhen, China). Since bats may differ in the amount of daily food intake, thus causing daily variation in body mass, we measured the body mass of each male for 10 consecutive days between 1900 and 2100 hours and used the mean value in our analyses.

Behavioural Recording and Analysis

After a week of acclimation, we used two infrared cameras (FDR-AX60, Sony, Tokyo, Japan) to record agonistic interactions among the 18 males for 17 consecutive days in the climatic chamber (Fig. S5). The two cameras were mounted 30 cm above the ground to monitor the bats' behaviour. The recordings were conducted from 1900 to 0700 hours the next day. We analysed videos using an IqiyiPlayer (Version 5.4.0.5375, Iqiyi Pictures, Beijing, China).

Dominance Hierarchy Determination

Following David (1987) and Gammell et al. (2003), we quantified the dominance hierarchy using David's scores (DS). The DS is calculated as $DS = w + w_2 - l - l_2$ where w represents the sum of the proportion of successes by focal individual i in his agonistic interactions with all other individuals and w_2 is the sum of w values for all individuals with which focal individual i interacted. Similarly, l represents the sum of proportion of failures by focal individual i in his agonistic interactions with all other individuals, and l_2 is the sum of l values for all individuals with which focal individual i interacted. Larger positive values of DS show higher dominance rank and larger negative values of DS show lower dominance rank. Following Sun et al. (2019), a winner was defined as the individual remaining at the location of an interaction after the withdrawal of the loser. A loser was identified as the individual leaving the location of interaction as an obvious result of fighting activity with a rival, and not conducting any aggressive behaviours or communication calls for at least 20 s after withdrawal. The time interval between two consecutive contests was at least 1 min.

Following Landau (1951), we calculated the Landau h value to test the linearity of the dominance hierarchy in male *H. armiger*. The h is calculated as $h = [12/(n^3 - n)] \times \sum[s_i - (n-1)/2]^2$ where n is the sum of individuals, and s_i is the number of individuals that individual i can beat in the whole group. The h value ranges from 0 (nonlinear order) to 1 (perfectly linear order; Appleby, 1983). We conducted randomization tests (10 000 randomizations) to determine the probabilistic significance of the linearity of the dominance hierarchies (de Vries, 1995). Linearity that is imperfect but has a

Table 1
Experimental schedule

Date	Events	No. of days	Bat marking method
20 November 2021 to 26 November 2021	Bat captures in the field and adaptation to the testing cage	7	See Table S1
27 November 2021 to 13 December 2021	Dominance hierarchy determination	17	See Table S1
14 December 2021 to 15 December 2021	Recovery and resting	2	18 bats: one band of each bat
16 December 2021 to 30 December 2021	Forehead gland secretion collection	15	18 bats: one band of each bat
31 December 2021 to 12 January 2022	Assays of corticosterone and testosterone	13	16 bats: one band of each bat; 2 bats: no band
13 January 2022 to 26 January 2022	Recovery and resting	14	16 bats: one band of each bat; 2 bats: no band
27 January 2022 to 31 January 2022	Assays of immunological indices	5	16 bats: one band of each bat; 2 bats: no band
01 February 2022 to 07 February 2022	Recovery and resting	7	16 bats: one band of each bat; 2 bats: no band
08 February 2022 to 23 February 2022	RMR monitoring	16	16 bats: one band of each bat; 2 bats: no band
24 February 2022 to 25 March 2022	Recovery and resting	30	All bands were removed
26 March 2022	All 18 bats were released in good health at their original site of capture	1	All bands were removed

significant *P* value shows a near-linear dominance hierarchy (de Vries, 1998). This was calculated using the function ‘compete’ in the R package ‘compete’ (Curley, 2016).

Forehead Gland Secretion Collection and Chemical Compounds Analysis

The forehead gland exudates were collected after the dominance hierarchy determination. The forehead gland extrudes a black gland substance. We extracted the black gland secretion by gently squeezing the area around the forehead gland and then transferring it into a 20 ml headspace glass vial with a PTFE-lined septum by presterilized forceps. All samples were collected from 1900 to 2000. To eliminate any effect of potential contaminants, we collected one blank sample by waving the vial three times in the air during each sampling period. Sampling was conducted every 5 days for each individual. Each male was sampled three times. All samples were immediately stored at −80 °C until analysis.

We did not collect useable samples from two of the 18 bats (bat.1 and bat.12) over each of the 5-day intervals due to inadequate secretion or a complete lack of extruded secretion for the sampling periods; therefore, we excluded these two males from all subsequent analyses.

Following previous studies (Zhang et al., 2022), we conducted the gas chromatography−mass spectrometry (GC−MS) analysis for compound identification. Before analysis, an internal standard (IS) of 10 μl of 2-octanol (10 mg/l stock in dH_2O) was added to each sample. All the mixed samples (i.e. IS and gland secretion) were heated at 60 °C for 15 min, and then, the samples were extracted for 30 min by headspace solid-phase microextraction (SPME) with 50/30 μm DVB/CAR/PDMS SPME fibre coating. Here, DVB is divinylbenzene, CAR is carboxen and PDMS is polydimethylsiloxane. After the extraction, the volatile compounds were desorbed from the SPME fibre coating and then inserted directly into the injector port at 250 °C.

The GC−MS analysis was conducted using an Agilent 7890 gas chromatograph system connected to an Agilent 5977 mass spectrometer with an EI ion source (70 eV). A DB-Wax capillary column (30 m × 250 μm inner diameter and 0.25 μm film thickness; Agilent) was used with helium (at 1 ml/min) as the carrier gas. One microlitre of the sample was injected in a 1:1 split mode. The purge flow rate of the front inlet septum was 3 ml/min. The column temperature was held at 40 °C for 4 min, then increased to 245 °C at the rate of 5 °C/min and then held at 245 °C for another 5 min. The temperature of the ion source, front injection and transfer line was 230 °C, 250 °C and 260 °C, respectively. The mass spectrometer was performed in the mode of full scan with an *m/z* range from 20 to 400 and a solvent delay of 0 min.

All chromatographic data, including measurement of raw peak, filtration of the data baseline, correction for baseline shift, analysis of deconvolution, peak alignment, identification, integration and spectrum match, were preprocessed in Chroma TOF version 4.3X and the National Institute of Standards and Technology database (Kind et al., 2009). We calculated the relative peak area using the peak area of each volatile compound divided by the peak area of IS in the same analytical run. We calculated the average of the relative peak area for all compounds of each category as the relative peak area for that category of compounds. In order to increase the accuracy of volatile compound identifications, we took the following three measures: (1) volatile compounds with less than 80% similarity compared with compounds in the National Institute of Standards and Technology database and the relative peak area less than 0.01% were excluded from further analyses (Burgener et al., 2009; Weiß et al., 2018), (2) we ran a control blank sample and removed the compounds that were considered as contaminants or exogenous compounds if the concentration of compounds in the blank sample is similar or higher than that in the gland secretion sample and (3) we removed the compounds that are extremely rare in natural products.

When we selected the volatile compounds with greater than 80% similarity, we found a total of 39 volatile compounds from the blank sample remained (Research Data S3), and these 39 compounds were not present in the 24 compounds from the gland secretions.

Assays of Corticosterone and Testosterone

An initial blood sample (80 μl) was collected from 16 bats between 1730 and 1830 hours into heparinized microhematocrit capillary tubes after puncturing the wing vein with a 26G hypodermic needle. All blood samples were collected within 3 min of opening the climatic chamber door to minimize the potential impacts of handling on plasma CORT levels. Then, the bats were placed into individual cloth bags, and subsequently, 80 μl blood samples were collected 30 min after capture for stress-induced CORT profiles of acute stress. All collected blood samples were immediately centrifuged at 3000 rpm for 10 min to separate plasma. Plasma was aspirated, split into two fractions, and stored at −80 °C until use. Sampling was performed every five days for each male. Each individual was sampled three times.

Plasma CORT and T concentrations were determined using enzyme-linked immunoassay kits (Cat No. ADI-901-097 and ADI-901-065, Enzo Life Sciences, Farmingdale, NY, U.S.A.) following the manufacturer's specifications. The optimal plasma dilution with steroid displacement buffer for CORT and T assays was 1:50 plasma with 2% steroid displacement buffer. The inter- and intraplate coefficients of variation were 4.9% and 1.3%, respectively. We excluded stress-induced CORT levels from subsequent analyses because baseline CORT levels strongly correlated with stress-induced CORT levels (Pearson correlation: $r_{16} = -0.902$; $P < 0.001$).

Assays of Immunological Indices

Assays of immunological indices were performed after the assays for CORT and T. Lipopolysaccharide (LPS) is a bacterial endotoxin that can simulate an infection and trigger an immune response in many taxa, including bats (Stockmaier et al., 2020). After collecting blood for the assays of hormone levels followed by a 2-week period of recovery, each male was injected with 65−92 μl of phosphate-buffered saline (PBS), which acted as a control. Twenty-four hours after PBS injection, the bats were injected with the same amount of LPS (L2880, Sigma-Aldrich, Israel) in PBS at a dosage of 5 mg/kg. Both injections occurred at the interfemoral membrane below the knee at the point of contact with the left leg. We collected a blood sample (70 μl) 24 hours after the injection of PBS and then after injection with LPS.

White blood cell counts

Blood smears were air-dried and stained with Liu's stain (Yue et al., 2014). All smears were investigated at 400× magnification to determine the proportion of different types of leukocytes (neutrophils, lymphocytes, basophils, eosinophils and monocytes) in the total leukocyte counts (100 counts) per smear with a complete monolayer of red blood cells (Cīrule et al., 2012; Walberg, 2001). We then calculated the neutrophil/lymphocyte (N/L) ratio by dividing the total number of neutrophils by the number of lymphocytes for each male. The difference in N/L ratio between the PBS injection and the LPS injection was used as the immune index.

Assay of IgG and IgM

Immunoglobulin G (IgG) and immunoglobulin M (IgM) are components of the adaptive humoral immune system and are the most abundant class of antibodies in mammalian blood (Kuby, 1997). They are widely used to measure immune function of animals including bats (Schountz, 2013). Plasma IgG and IgM levels were determined with enzyme-linked immunosorbent assay kits following the manufacturer's instructions (IgG, Cat No. YJ791593; IgM, Cat No. YJ791502, Shanghai, China). Briefly, 10 μl raw plasma was directly diluted with 40 μl of Sample Diluent Buffer (0.05 M PBS) in a flat-bottomed enzyme-labelled 96-well plate. After 30 min of incubation at 37 °C, each well was washed five times with 200 μL Wash Buffer (PBST with 0.15% Tween-20). Fifty microlitres of the detecting horseradish peroxidase-conjugated anti-rat IgG or IgM were then added to each well and incubated for another 30 min at 37 °C and then washed again. After adding mixed chromogenic solution (combined horseradish peroxidase catalysis 3,3′,5,5′-tetramethylbenzidine) and stop solution (2 M sulphuric acid), the optical density of each well was read at 450 nm wavelength using a microplate spectrophotometer. The levels of IgG (mg/ml) and IgM (mg/ml) were calculated according to the five dilutions of the standard curves, respectively. All samples were run in duplicate across two 96-well plates. Intra- and interassay variations for IgG and IgM were 8.0% and 11.4%, 7.9%, and 9.9%, respectively. The difference in IgG and IgM levels after PBS injection and LPS injection was used as a second immune index in addition to the N/L ratio.

Resting Metabolic Rate Monitoring

Resting metabolic rate (RMR) has been used as a proxy for low energy availability (Kinoshita et al., 2021). RMR of 16 bats was measured using a Fox Box Respirometry System (FOX-BOX, Sable Systems, 255 Las Vegas, NV, U.S.A.), connected to a cylinder breathing chamber (radius: 15 cm; height: 30 cm; 4.5 L). Bats were fasted for at least 12 h prior to measurement of RMR to minimize the effects of food digestion on metabolic rates. The experiments were carried out in a climatic chamber (length × width × height: 2.1 × 1.8 × 2.2 m) at a temperature around 22 °C. Briefly, each bat was weighed and placed into a cylinder breathing chamber. All measurements of RMR were performed between 1200 and 1800 hours. Within this time interval, the carbon dioxide production and oxygen consumption of each individual were recorded for a continuous period of 3 h. Airflow rate was 550 ml/min. Data were collected every 5 min. The RMR was expressed as ml/min/g. The RMR is calculated as $\mathrm{RMR} = [\mathrm{FR} \times ((\mathrm{FiO_2} - \mathrm{FeO_2}) - \mathrm{FeO_2} \times (\mathrm{FeCO_2} - \mathrm{FiCO_2}))/(1 - \mathrm{FeO_2})]/\mathrm{BM}$ where FR represents the airflow rate (ml/min), $\mathrm{FiO_2}$ and $\mathrm{FeO_2}$ are the oxygen concentration in the incoming and outgoing air, respectively, $\mathrm{FiCO_2}$ and $\mathrm{FeCO_2}$ are the carbon dioxide concentration in the incoming and outgoing air, respectively, and BM is the body mass in g. Each individual was measured every 7 days and was measured three times. The mean RMR value was used for analysis.

Statistical Analysis

We used individual average values of all parameters including physical condition, hormone levels, immunocompetence and RMR, except for the olfactory parameters used for the individual signature. We conducted Kolmogorov−Smirnov tests to examine the normality of all our data and found that all variables met a normal distribution ($P > 0.05$). We thus used a Pearson correlation test to examine the relationships between physical condition, dominance rank and physiological quality.

The relative peak area of the GC−MS peaks was calculated using the peak area of each compound divided by that of the peak area of the IS. We calculated the Bray−Curtis similarity index based on the relative peak area between each pair of samples, which was then used in a nonmetric multidimensional scaling ordination. The nonmetric multidimensional scaling plot placed each sample in a two-dimensional space so that the relative distance between samples matched their chemical similarities. We used ‘stress’ to measure goodness of fit, which evaluated how well a specific configuration reproduced the observed distance matrix related to the data. We adopted the following standards for stress results: stress < 0.05 presented an excellent model in a two-dimensional space, $0.05 <$ stress < 0.1 was very good, $0.1 <$ stress < 0.2 was good and stress > 0.2 presented a poor model in a two-dimensional space (Kruskal, 1964).

To compare the difference in chemical compounds of gland secretions among the 16 individuals, we calculated a nonparametric analysis of similarity (ANOSIM) using 1000 permutations based on the Bray−Curtis similarity distance matrix of each individual. ANOSIM tests are a series of Mantel-type permutations or random programs that make no assumptions about the distribution of the data (Clarke & Warwick, 2001). Global *R* is defined as the difference in mean rank dissimilarity within groups compared with between groups. When Global R is closer to 1, samples from the same individual are more similar to one another and more dissimilar to samples from other individuals.

To test the difference in categories of compounds of gland secretions among the 16 individuals, we calculated the Bray−Curtis similarity index based on the mean relative peak area of each category of compounds using 1000 permutations in the ANOSIM test procedure.

To test whether physical condition (BCI), dominance rank and physiological quality (CORT, T, IgG, IgM, N/L, RMR) could predict the olfactory parameters of the bat forehead gland secretion, we first performed a principal component (PC) analysis on the individual average values of the olfactory parameters. We extracted six PCs (with eigenvalues > 1) that explained 86.29% of the total variance (Table S3). We only selected the first three PC factor scores for subsequent analyses because the first three PCs explained 67.56% of the total variance, and the loadings of the first three PCs were higher than the remaining three PCs (Table S3). Subsequently, we performed linear models using the function ‘lmer’ in the R package ‘lmerTest’ (Kuznetsova et al., 2017). In this model, physical condition (BCI), dominance hierarchy and physiological quality (CORT, T, IgG, IgM, N/L, RMR) were used as independent variables, and each of the first three PC factor scores was used as a dependent variable. We also calculated the variance inflation factor of the independent variables to determine whether a linear model was applicable. Variance inflation factor was < 5 for all independent variables, indicating that a linear model could be used to test the null hypothesis. The model generated a set of 256 candidate models, including the main effects of the independent variables and all possible combinations of these main effects via linear regressions. The residuals of all models met a normal distribution (Kolmogorov−Smirnov; PC1: $0.68 < P < 0.99$; PC2: $0.23 < P < 0.88$; PC3: $0.57 < P < 0.99$).

In all cases, we selected the best model using the Akaike information criterion corrected for small sample size (AICc) using the function ‘dredge’ in the R package ‘MuMIn’ (Bartoń, 2022). The model with the lowest AICc value indicates the best-fitting model. Differences among AIC values were calculated as follows: $\Delta_i = \mathrm{AIC}_i - \mathrm{AIC}_{\min}$. Furthermore, $\Delta\mathrm{AICc} > 2$ between the first and the second-best models is considered the gold standard for model selection (Burnham & Anderson, 2002); therefore, multimodel inference was performed if AIC differences were ≤ 2, using the ‘model.avg’ function in the package ‘MuMIn’ (Bartoń, 2022). Akaike weights (*wi*) were also calculated to explain the relative likelihood of a given model; these values represent the normalization of the probabilities of different models, given the data (Lukacs et al., 2007). The significance of the results was assessed by whether the 95% CIs overlapped zero.

A hierarchical partitioning (HP) analysis was performed to explain the independent effect of each independent variable (Chevan & Sutherland, 1991). This analysis estimated the independent contribution of each independent variable, which weakens multicollinearity among predictor variables (Graham, 2003). The HP analysis was conducted using the function ‘hier.part’ in the R package ‘hier.part’ (Mac Nally & Walsh, 2004). In the HP, we assessed the statistical significance of each independent variable using a randomization method (Mac Nally & Walsh, 2004). All statistical tests were conducted in R v. 4.1.2 (R Core Development Team, 2021).

RESULTS

Scent Profiles

We detected a total of 432 volatile compounds in 48 samples from 16 individuals (mean ± SE = 6.94 ± 0.38 mg/sample; range 5–10 mg; Fig. S6). A total of 24 volatile compounds remained after filtering and these compounds were used for further statistical analyses (Research Data 1). Detected volatile compounds were classified into the following seven categories: alcohol (25%), ketone (21%), aromatic compounds (17%), ester (17%), aldehyde (8%), alkane (8%) and carboxylic acid (4%).

Individual-Specific Scent Profile

There were significant differences in chemical composition between individuals based on the relative peak area of each volatile compound (ANOSIM: global $R = 0.427$, $P = 0.001$; Fig. 1a). Based on the average of the relative peak area of each category of compounds, there were also significant differences in categories of compounds between individuals (ANOSIM: global $R = 0.373$, $P = 0.001$; Fig. 1b). Individually specific scent profiles in samples are presented in Table 2.

Dominance Rank, Physical Condition, Hormone Levels, RMR and Immunocompetence

A total of 2089 agonistic interactions from 18 bats (mean ± SD: 116.06 ± 48.77 interactions per bat; range 48–218) were used to estimate dominance rank (Fig. S7). The DS for all 18 male *H. armiger* are shown in Table S4. There was at least one agonistic interaction between each pair of males. Among the 2089 interactions, the mean ± SD contest duration was 18.62 ± 19.43 s (range 2–276 s). Male *H. armiger* formed a near-linear dominance hierarchy ($h = 0.674, P < 0.001$).

Table 2
Description of the number of compounds in individual *Hipposideros armiger* gland scent profiles with three repeat samples

Bat ID	Mean no. of compounds per bat ± SE	No. of compounds occurring in all samples from the bat	No. of samples
Bat.2	22.33 ± 1.67	19	3
Bat.3	18.33 ± 1.45	16	3
Bat.4	20.33 ± 1.86	18	3
Bat.5	20.67 ± 0.67	17	3
Bat.6	19.67 ± 0.88	18	3
Bat.7	22.67 ± 0.67	21	3
Bat.8	21.33 ± 1.33	20	3
Bat.9	22.00 ± 1.00	19	3
Bat.10	21.00 ± 0.01	21	3
Bat.11	21.00 ± 0.01	21	3
Bat.13	22.00 ± 0.58	20	3
Bat.14	19.67 ± 0.33	19	3
Bat.15	18.33 ± 0.33	17	3
Bat.16	19.33 ± 0.88	18	3
Bat.17	23.67 ± 0.33	23	3
Bat.18	21.00 ± 0.01	21	3

The mean ± SD of dominance ranks, body condition measures, hormone levels, RMR and immunocompetence are shown in Table 3. The abbreviations of each of the variables are also listed in Table 3. All raw data can be found in Research Data 2.

DS was negatively correlated with both CORT and T (Table 4), and CORT and T were in turn positively correlated with each other. BCI correlated positively and significantly with T (Table 4). Besides the T/CORT correlation, there was one other significant correlation between physiological measures: RMR correlated positively and significantly with T (Table 4). No other correlations were significant.

Olfactory PCs, Dominance Status, Physical Condition and Physiological Quality

N/L was the only independent variable that was retained in the best AICc model for PC1 (Table S5). Model averaging revealed that PC1 was significantly and positively associated with N/L (Fig. 2a; Table S6). Moreover, HP showed that N/L (33.88%) predicted significantly more variation in the PC1 compared with the other independent variables (ranging from 4.46% to 19.75%; Fig. 2c; Table S6). Thus, males with higher N/L had secretions with relatively higher proportions of one alcohol, one aldehyde, two alkanes, one carboxylic acid, two esters, two ketones and two aromatic compounds and lower proportions of two alcohols, one ester and one ketone (Table S3).

The best AICc model for PC3 contained two independent variables, that is, T and DS (Table S5). Model averaging showed that PC3 was not significantly associated with T while significantly and negatively associated with DS (Fig. 2b; Table S6). Furthermore, HP showed that DS contributed significantly more (36.94%) to the variation in PC3 than other independent variables (ranging from 2.91% to 15.97%; Fig. 2d; Table S6). Therefore, higher-ranking males had secretions with relatively lower proportions of two alcohols, one aldehyde and two aromatic compounds (see Table S3).

PC2 was not significantly correlated with any independent variables (Tables S5 and S6).

DISCUSSION

In this study, we found pronounced individual differences in the concentrations and categories of compounds from the forehead gland, which supported our first hypothesis that olfactory signals of male *H. armiger* can encode individual signatures. We also found no significant relationship between BCI and olfactory parameters, which failed to support the second hypothesis that olfactory signals predict signallers' physical condition. Moreover, we found that the PC3 factor scores of olfactory parameters were positively associated with David's score, supporting the third hypothesis that olfactory signals predict signallers' dominance status. In addition, we found that many olfactory parameters were significantly related with N/L ratio. These results partially supported the fourth hypothesis that olfactory signals predicted individuals' physiological quality. Finally, we found that male *H. armiger* established a near-linear dominance hierarchy, thus validating the use of our dominance scores in our analyses. To our knowledge, this is the first experimental evidence that olfactory signals of bats can encode multiple types of information in the context of territorial conflicts.

Table 3
Nomenclature and abbreviations of variables, and summary of mean ± SD and range of each variable in *Hipposideros armiger*

Variable abbreviation	Variable name	Mean	SD	Minimum	Maximum
DS	David's score	−3.89	50.66	−106.02	85.14
BCI	Body condition index	0.067	0.07	0.55	0.76
CORT	Baseline corticosterone level (ng/ml)	14.09	14.58	2.74	50.16
T	Testosterone level (ng/ml)	32.64	20.28	8.53	93.43
RMR	Resting metabolic rate (ml/min/g)	174.40	36.88	117.17	257.70
IgG	Immunoglobulin G level (μg/ml)	82.54	22.43	45.03	114.40
IgM	Immunoglobulin M level (μg/ml)	2.90	0.72	0.95	3.95
N/L	Neutrophils/lymphocyte ratio	2.98	2.13	0.04	7.62

Table 4
Pearson correlation coefficients (*r*; above diagonal) and corresponding *P* values (below diagonal) for the nine characteristic variables from 16 male *Hipposideros armiger*

Variable	DS	BCI	CORT	T	RMR	IgG	IgM	N/L
DS		0.051	−0.834	−0.854	−0.415	−0.125	−0.057	−0.196
BCI	0.852		−0.166	0.499	0.217	−0.101	−0.352	0.451
CORT	**0.001**	0.539		0.902	0.340	0.270	0.049	0.032
T	**0.001**	**0.049**	**0.001**		0.581	0.159	−0.064	0.189
RMR	0.110	0.419	0.197	**0.018**		−0.187	−0.256	0.198
IgG	0.645	0.710	0.312	0.555	0.488		0.022	0.033
IgM	0.835	0.181	0.857	0.814	0.339	0.935		−0.133
N/L	0.467	0.079	0.907	0.484	0.462	0.902	0.622	

Significant *P* values are given in bold type. See Table 3 for detailed name and abbreviation adopted for each variable.

Olfactory Signatures

We found significant individual signatures encoded in olfactory signals of male *H. armiger*, consistent with previous analyses (Zhang et al., 2022). Similar findings have been found in northern-shouldered bat, *Sturnira parvidens* (Faulkes et al., 2019), Bechstein's bat, *Myotis bechsteinii* (Safi & Kerth, 2003), and greater sac-winged bats, *Saccopteryx bilineata* (Caspers et al., 2008). Selective pressures may drive animals to clearly convey their individual identity to conspecifics in a complex social system because animals living in groups with complex social systems need more complex communication signals (e.g. increased individual-specific information; Blumstein & Armitage, 1997). *Hipposideros armiger* is a highly social species with a polygamous mating system (Yang, 2011). They often gather in caves in colonies of hundreds of individuals (Sun et al., 2018). Individual-specific odours in *H. armiger* could facilitate rapid discrimination among close rival neighbours, thus enabling appropriate conciliatory or agonistic responses to encountered conspecifics. Indeed, our previous study confirmed that *H. armiger* can discriminate among individuals based on olfactory signals (Zhang et al., 2022).

Physical Condition and Olfactory Parameters

A signal can be honest if its reliability is maintained by signal production or maintenance costs because only high-quality signallers can bear the high signalling cost (Zahavi, 1975). We found no significant correlation between BCI and olfactory parameters of male *H. armiger*. These results showed that olfactory signals of male *H. armiger* did not convey information on the physical condition of senders, suggesting that olfactory signals were not an honest index or a handicap signal. One possible interpretation is that the primary costs for volatile compounds are the maintenance costs of the specialized organs used for scent glands, rather than the minute amounts of volatile compounds produced (Wyatt, 2014). We suggest that the costs related to the maintenance of the scent glands of male *H. armiger*, including the cellular machinery, energy consumption during secretion, and upkeep of specialized tissues, are significant, whereas the synthesis of volatile compounds is relatively low cost. Future work should be performed to measure the oxygen consumption of the scent glandular tissue by stimulating or inhibiting glandular activity and thus quantify the maintenance costs of the scent glands.

Dominance Status and Olfactory Parameters

We found that male *H. armiger* formed near-linear dominance hierarchies. Similar results can be found in many species, such as insects (Peeters et al., 1999), fishes (Chase et al., 2002), birds (Devost et al., 2016), monkeys (Wittig & Boesch, 2003) and bats (Liu et al., 2020). The establishment of linear dominance hierarchies may be affected by two factors: availability of resources and a high frequency of fights within a group (Archie et al., 2006). First, limited resources may drive animals to live in a despotic society with strong linear dominance hierarchies. This is because individuals with a high dominance status can gain priority access to more high-quality resources (Archie et al., 2006; Wrangham, 1980). Second, animals involved in frequent within-group conflicts that have a high risk of injury are more likely to form linear dominance hierarchies to minimize the costs of fighting (Rowell, 1974). Male great Himalayan leaf-nosed bats normally maintain a minimum distance of 10−15 cm from their conspecifics and often fight to defend their roost territory (Sun et al., 2018). The roost territory is a very important resource for male *H. armiger* because during the breeding season, males can gain more mating opportunities by protecting females against other males in their roost territory (Yang, 2011). As a result, limited roost resources and frequent conflicts among individuals may explain why male *H. armiger* establish near-linear dominance hierarchies.

The maintenance of dominance relationships within groups can depend on the ability of a species to communicate and show their high dominance rank through a wide range of chemosensory cues (Fernald, 2014). We found that PC3 showed a significant and negative relationship with David's score (DS), meaning that male *H. armiger* with higher dominance ranks produced secretions with relatively low proportions of some alcohols, aldehydes and aromatic compounds. However, the estimated effect size of the DS in PC3 is small, that is, −0.012. This may be because the relationship between DS and PC3 might be dominated by other variables such as BCI or T, which have larger effects on PC3, thus reducing the relative influence of DS. In total, these results indicate that the forehead gland secretions of male *H. armiger* may signal relative dominance rank. A similar result has been documented in male mandrills, *Mandrillus sphinx* (Setchell et al., 2010), male blackbucks, *Antelope cevicapra*, (Rajagopal et al., 2010) and European rabbits, *Oryctolagus cuniculus* (Hayes et al., 2003).

Appropriate marking methods play a critical role in ensuring the accuracy, ethical integrity and scientific validity of behavioural research. In the present study, among the 18 bats, four (22%) had

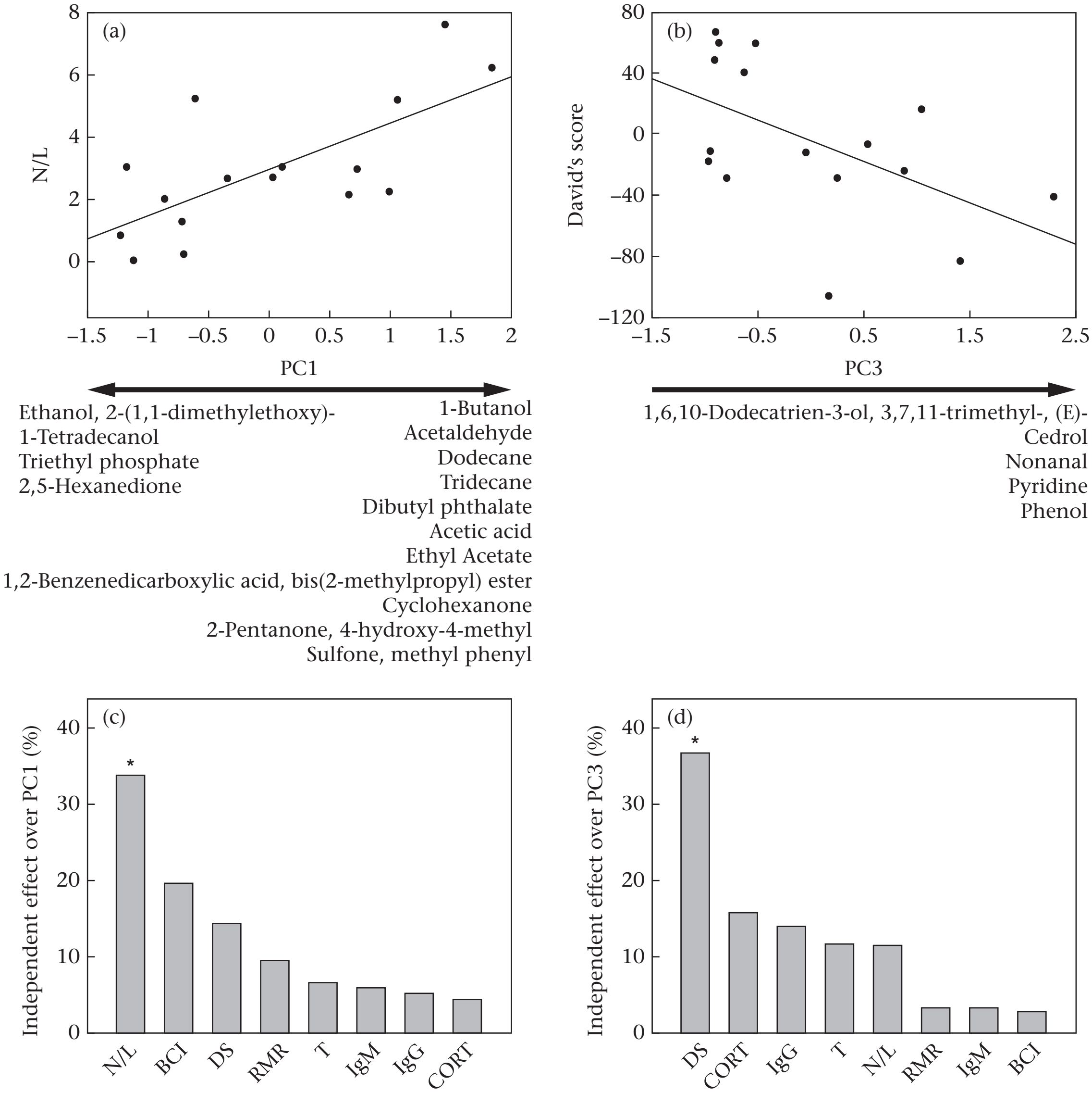

Figure 2. Relationships between PC scores from chemical analysis versus physical condition measures and dominance measures for *Hipposideros armiger*. (a) N/L as a function of PC1, (b) David's score as a function of PC3 and (c) and (d) present independent effects (as percentages) of the eight values of physical condition and dominance measurements for PC1 and PC3, respectively, calculated with HP (see text). The arrows under the scatter plots indicate the relationships of the PC scores with the relative proportions of determined compounds. See Table 3 for detailed name and abbreviation adopted for each variable. $*P < 0.05$. $N = 16$.

four or five bands on their forearms and 14 (78%) had three or fewer bands on their forearms. There were no significant differences in the mean fight duration and the total number of fights between the abovementioned two sets of marked bats (independent sample *t* test; fight duration: $t_{16} = 0.394$, $P = 0.699$; number of fights: $t_{16} = 0.885$, $P = 0.389$). These results suggest that the marking procedure may not significantly influence the natural behaviours of bats and thus the establishment of dominance hierarchies. However, alternative marking methods, such as with one colourful plastic band per individual, should be used for future studies to minimize the influence of marker rings on the natural behaviour of bats.

Physiological Quality and Olfactory Parameters

Immunocompetence

We found that male *H. armiger* with better immunocompetence as indicated by N/L had significantly higher proportions of some alcohols, aldehydes, alkanes, carboxylic acids, esters, ketones and aromatic compounds in their secretions. These findings imply that olfactory signals of male *H. armiger* may relay information concerning the individual's immunocompetence. One possibility may account for this phenomenon. Because olfactory signals of male *H. armiger* may play a role in mate attractors, given the sexual dimorphism of the glands in this species, a female's preference for male olfactory signals may be dose-dependent, and thus, it may be possible that males with better immunocompetence could simply produce more specific chemical compounds than those males with lower immunocompetence. A similar phenomenon can be found in the mealworm beetles, *Tenebrio molitor* (August, 1971). However, further behavioural experiments are needed to examine whether female *H. armiger* prefers olfactory signals from males with better immunocompetence.

Hormonal levels

We found no significant relationship between olfactory parameters and plasma T levels and plasma CORT levels, indicating that the forehead gland secretions of male *H. armiger* may not encode information about the hormonal levels of the signallers.

Because plasma T and CORT levels correlated with the dominance rank of male *H. armiger* and lower-ranking males tend to be individuals with higher plasma CORT and T levels, encoding hormonal information in olfactory signals may thus not be beneficial for low-ranking males.

This raises an additional question: why do low-ranking male *H. armiger* have higher plasma CORT and T levels than high-ranking males. One possible interpretation for the negative correlation between dominance rank and CORT levels is that suppression of behaviours as a result of acts of dominance by higher-ranking individuals may become a chronic stressor for subordinates, and chronic social stress might increase the release of CORT in subordinates by excessive activation of the hypothalamic—pituitary—adrenal axis (Sapolsky, 2005). For example, low-ranking rhesus macaques, *Macaca mulatta tcheliensis*, have higher cortisol levels compared with more dominant individuals (Zhang et al., 2018).

Previous studies showed that dominance rank affects T across many vertebrate species (Wingfield et al., 1990). Low-ranking males often have relatively low T levels compared with higher-ranking individuals (Muehlenbein et al., 2004; Muller & Wrangham, 2004). In contrast, low-ranked males displayed higher T levels than higher-ranked males in this study. A similar phenomenon has been documented in red deer males, *Cervus elaphus* (Bartoš et al., 2010), and rock hyraxes, *Procavia capensis* (Koren & Geffen, 2009). Age is a possible factor that might explain the inverse relationship between dominance rank and T in male *H. armiger*. Testosterone levels decrease with age in human males (Bribiescas, 2001), and some high- and middle-ranking male *H. armiger* in our sample may be relatively old because they had a high degree of tooth wear of the upper canines and premolars relative to low-ranking males (C. Sun, personal observation). Further research will be needed to explore this possibility.

Resting metabolic rate

RMRs may indicate an individual's capacity to perform well during challenging activities, and thus, only individuals that could maintain higher self-maintenance costs could afford to spend energy in additional activities (Daan et al., 1990; Biro & Stamps, 2010). For example, male American goldfinches, *Spinus tristis*: individuals with a higher resting metabolic rate have more colourful plumage ornaments (Kelly et al., 2012). However, olfactory parameters did not predict the resting metabolic rates of male *H. armiger* in this study. This illustrates that the forehead gland secretions of male *H. armiger* may not provide information on the resting metabolic rates of the signallers. One possible interpretation is that the production of these volatile compounds in male *H. armiger* may not require significant energy consumption. Male *H. armiger* also performed wing flapping and uttered territorial calls during territorial defence for day-roost (Sun et al., 2018). In this case, it is reasonable to assume that male *H. armiger* may spend more energy in wing flapping and/or vocalizations than in odour production. Future studies should integrate multimodal behaviours and compare their differences in energy consumption.

Limitations of the Study

We acknowledge that our study had three limitations. First, we believe that age may be one factor in the establishment of a dominance hierarchy. However, we cannot accurately estimate the age of adult bats based on forearm length because the forearm length of adult bats does not change-over time. Future work should be conducted to accurately assess the age of the adult bats using DNA methylation (Wilkinson et al., 2021). Second, although our results suggested that olfactory signals of *H. armiger* may encode multiple types of information, further investigations are needed in the field to investigate our findings' generality, and further behavioural experiments are needed to examine whether males can both perceive and use the compounds of an opponent's forehead gland scent reflecting different physiological quality or dominance rank to make decisions during aggressive interactions. Third, we acknowledge that for some compounds, the spectral data may not be sufficient for a confident identification. Further work is needed to enhance compound identification through the use of additional techniques, such as standards comparison, high-resolution mass spectrometry and more advanced cheminformatics tools.

Conclusion

Our results demonstrate that olfactory signals of male *H. armiger* provide honest information about individual identity, dominance rank and physiological quality. Further behavioural experiments will need to investigate the effects of secretion on the dominance or linear hierarchy of bats before and after the exudation. To our knowledge, this is the most detailed study to date concerning different information potentially encoded in olfactory signals in a bat species. We hope our findings will be helpful to other researchers who wish to investigate the design and evolution of olfactory signals in bats.

Author Contributions

Chunmian Zhang: Writing — review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chengbo Zhuang:** Methodology. **Congnan Sun:** Writing — original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dongming Li:** Writing — review & editing, Funding acquisition. **Jeffrey R. Lucas:** Writing — review & editing. **Limin Wang:** Methodology. **Jinpu Li:** Methodology. **Yang Wang:** Methodology.

Data Availability

The data on which this study is based are available in the Supplementary Material.

Declaration of Interest

We declare that we have no competing interests.

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Supplementary Material

Supplementary material associated with this article is available at https://doi.org/10.1016/j.anbehav.2025.123227.

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