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Individuality and function of chemical signals during conflict resolution of a mammal

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Individual recognition via communication signals is a critical component of social behavior, and provides the basis of conflict resolution, territorial behavior, and mate choice. However, the function of chemical signals in mammalian individual recognition and conflict resolution has largely been unexplored despite olfaction being a dominant sensory modality in many mammalian species. Here, we describe behavioral tests designed to evaluate the potential role of forehead gland secretions during conflict related to territorial defense in male Great Himalayan leaf-nosed bats. We used gas chromatography–mass spectrometry to quantify the chemical composition. Our results showed that forehead gland secretions contain 16 categories of compounds, including 84 volatile compounds. The concentrations of compounds and their categories differed significantly among individuals. Moreover, behavioral studies indicated that males can use chemical signals for individual recognition. Contests were staged between males with or without functioning forehead glands. Paired males without functioning forehead glands displayed more physical contact and longer contest duration compared with pairs with functioning glands. Moreover, males with a functioning gland were more likely to win in contests when paired with males without a functioning gland. These findings support a growing amount of evidence that chemical signals play a vital role in conflict resolution in mammals.

Keywords: agonistic interaction; bats; chemical communication; conflict resolution; individual discrimination

Introduction

Contests over limited resources are ubiquitous in the animal kingdom.[1] However, contests may increase injury risk and generate significant energy costs, and may result in fatalities.[2,3] Therefore, most animals tend to resolve a conflict by exchanging information via communicative signals about their fighting ability, aggressive motivation, or social status before engaging in costly physical contests.[1] Individual recognition can also be a critical element in the information exchanged during these conflicts.[4] The ability to recognize opponents may facilitate subsequent interactions through decreased costly agonistic competition between individuals with high fighting ability.[1]

Most of the studies on the role of signals in conflict resolution have focused on visual and acoustic signals.[5,6] How olfactory signals affect conflict resolution is less well-known because the integration of advanced chemical analytical techniques and behavioral discrimination assays has been underutilized to date.[7] However, there is a renewed

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interest in the importance of the information encoded in chemical signals and their potential function[8]—a subdiscipline now called ethochemistry.[9]

Olfaction is one of the dominant sensory modalities in many mammalian species, and chemical signals secreted by conspecifics have been shown to markedly affect behavior.[10] Indeed, chemical signals play a crucial role in many social behaviors, including individual recognition,[11] territory defense,[12] and predation risk assessment.[8] For example, in bats, chemical signals play an important role in individual or species discrimination, parent–offspring recognition, courtship, and territorial scent marking.[13–21] However, studies on mammalian chemical communication used in aggression contexts have largely focused on territorial scent marking.[10] Little is known about the role of chemical signals during agonistic interactions over territories in vertebrates, especially in nocturnal mammals.

Here, we employed the framework of ethochemistry to investigate the role of chemical signals in agonistic interactions over territories in the Great Himalayan leaf-nosed bat (*Hipposideros armiger*). *H. armiger* is a nocturnal and highly gregarious species that usually roosts in caves, sharing day and night roosts among hundreds of individuals.[22] Our previous studies[23] showed that adult males defend their private roosting territory using conspicuous agonistic displays (Table S1, online only; description of behavioral terms follows Clement *et al.*[24] and Fernandez *et al.*[25]) and that territorial calls (Fig. S1, online only) convey information about individual identity and emotional state.[26] During territorial defense, male *H. armiger* also emit a pungent odor from a thick black secretion via an active protrusion from the forehead gland (Fig. 1A; Video S1, online only). This secretion can be detected by humans within a distance of 20 cm (C.Z., personal observation). Taken together, these observations suggest that chemical signals in *H. armiger* may play some role in territorial conflict resolution.

We integrated chemical composition analysis and behavioral assays to investigate individual variability in the composition of the forehead secretion and the possible function of this chemical signal in territorial conflict resolution in *H. armiger*. First, since individual recognition is a prerequisite for almost all social interactions,[27] we hypothesized that chemical signals in *H. armiger* could encode individual identity information and would facilitate individual recognition. We thus predicted that (1) specific chemical compounds and their concentrations, as well as certain categories of compounds (e.g., alkanes versus alcohols) would vary among individuals, and (2) *H. armiger* males would have the ability to discriminate between individuals based on the chemical composition of the forehead secretion. Second, if bats could recognize individuals based on these chemical signals, we further hypothesized that the chemical signals would play a role in territorial conflict resolution. We predicted that (1) the proportion of physical contact and contest duration would increase when the odors were absent during territorial conflicts relative to when odors were present during the conflicts, and (2) individual *H. armiger* emitting an odor via gland protrusion would be more likely to win in fights when paired with bats with a disabled gland.

Materials and methods

Animals and housing in the laboratory

In April 2018, 17 adult *H. armiger* males were caught from the Shiyan cave in Chongyi, Jiangxi Province, China. The bats were housed in a husbandry room (6.5 m long × 5.5 m wide × 2.1 m high). The room was maintained at a temperature of around 23 °C, a relative humidity of around 60%, and a 12-h dark/light cycle. All bats were fed with *ad libitum* freshwater and larvae of *Zophobas morio* enriched with vitamins and minerals. All bats were marked with metal rings (4.2 mm; Porzana Ltd, East Sussex) on their forearm to identify individuals.

Animal husbandry and experimental procedures adhered to the Guidelines for the Use of Animals in Research (ASAB/ABS, 2021) and to the National Natural Science Foundation of China for experiments involving vertebrate animals and were approved by the Northeast Animal Research Authority of Northeast Normal University, China (approval number: NENU-W-2008-108). There were no deaths during the entire experimental period. After completing the experiments, all the bats were returned to their original caves.

Forehead gland secretion collection

The forehead gland forms a deep pocket above the nose that can be everted when palpated (Fig. 1A). We gently extruded the black gland secretion by

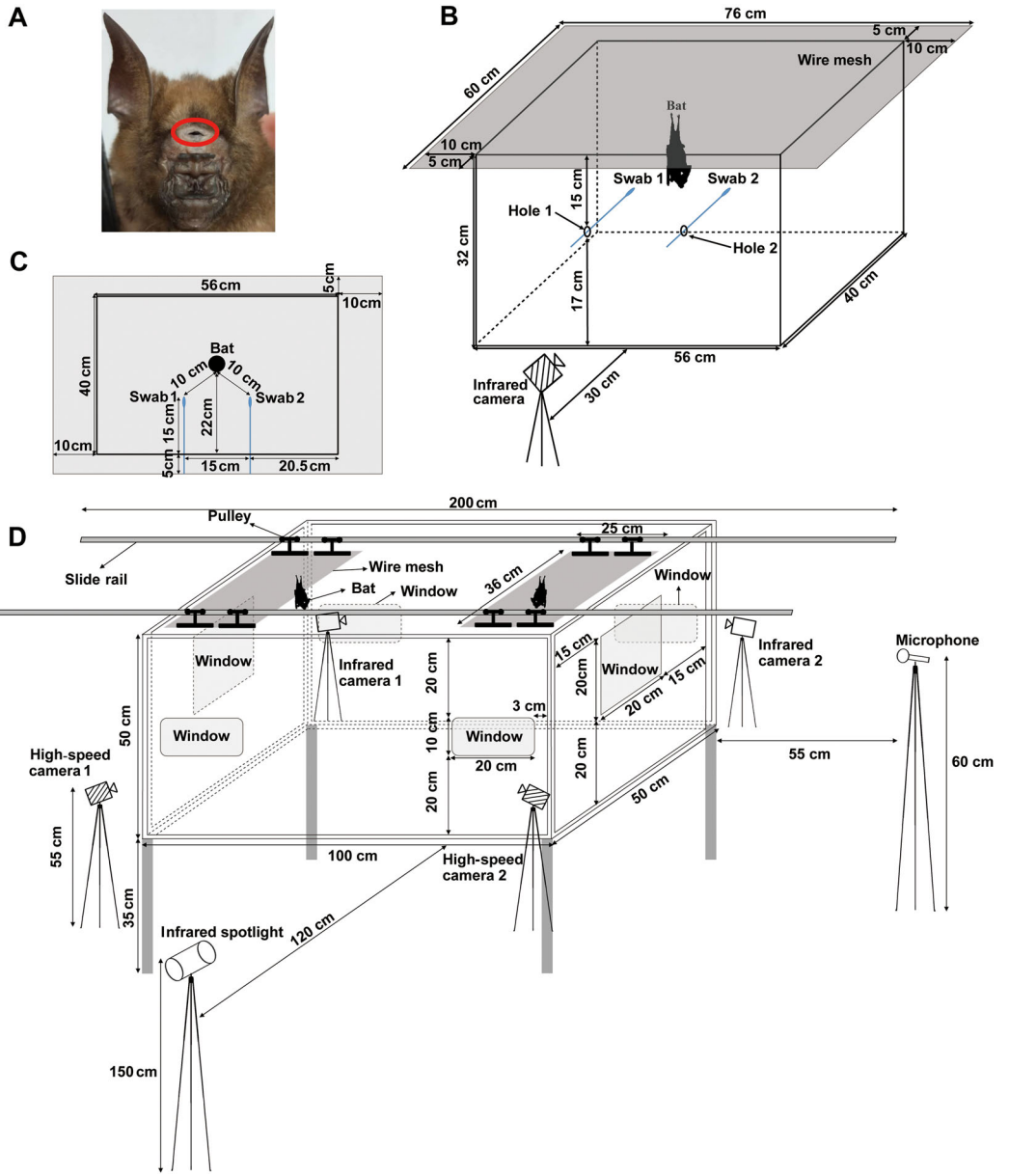

Figure 1. The forehead gland and experimental design. (A) Adult male *Hipposideros armiger*. The red oval indicates the location of the forehead gland. (B) The habituation–discrimination test apparatus. Two swabs were used for presenting odor samples. In the habituation phase, the odor source of swab 1 was the habituation odor, and swab 2 was used as a blank control. In the discrimination phase, the odor source of swab 1 was the novel odor, and the odor source of swab 2 was the habituation odor. (C) Top-down view of the habituation–discrimination tests. The distance between the tested bat and each swab was 10 centimeters. Before the trial, the wire mesh could be rotated so that the tested bats were equidistant from the two odor sources. (D) Gland protrusion manipulation apparatus. The experiments were conducted in a 1.00 m × 0.50 m × 0.50 m box made of acrylic sheet plexiglass. The lid was removed. There were two windows in the front and back to record the behavior of bats using infrared cameras, and one window on the left and right to record the calls of bats using a microphone. Four pulleys slide steadily on two rails. Before the trial, the two bats roosted in the center of two pieces of mesh (gray area).

squeezing the area around the forehead gland, and transferred it into a 20-mL glass headspace vial with a PTFE-lined septum using presterilized forceps. We collected all samples between 19:00 and 19:30. To exclude the effect of potential contaminants, one blank sample (i.e., ambient air) was collected by waving the glass headspace vial three times in the air during each sampling period.

We collected 33 samples from seven individuals (mean $\pm$ SE $=$ 4.7 $\pm$ 0.5 samples/individual; range: three to seven samples/individual). We attempted to collect seven sequential samples per individual to test for within-individual variation in secretion properties over time. Sampling was performed every 15 days for each individual because bats typically replenish gland secretion about 15 days after palpation (C.Z., personal observation). However, we were unable to collect a usable sample from each individual every 15 days due to inadequate secretion or a complete lack of extruded secretion for some of the sampling periods.

Chemical compounds analysis

Before all samples were analyzed, 10 μL of 2-octanol (10 mg/L stock in dH_2O) was added as an internal standard (IS). The mixed sample was heated for 15 min at 60 °C and then each sample was extracted for 30 min in headspace solid-phase microextraction (SPME) using 50/30 μm DVB/CAR/PDMS SPME fiber coating. After the volatile compounds were extracted, they were desorbed from the SPME fiber coating and then immediately inserted at 250 °C into the injector port.

The gas chromatography–mass spectrometry (GC-MS) analyses were performed with an Agilent 7890 gas chromatograph system linked to an Agilent 5977 mass spectrometer with the EI ion source (70 eV). The system utilized a DB-Wax capillary column (30 m $\times$ 250 μm inner diameter and 0.25-μm film thickness; Agilent). A 1-μL sample was injected in a 1:1 split mode. GC-MS analyses were performed with helium (at 1 mL/min) as the carrier gas. The front inlet septum purge flow was 3 mL/min. The initial temperature was kept at 40 °C for 4 min, then increased to 245 °C at a rate of 5 °C/min, and then kept at 245 °C for 5 minutes. The front injection, transfer line, and ion source temperature was 250, 260, and 230 °C, respectively. The mass spectrometry was conducted in the full-scan mode with an m/z range of 20–500 and a solvent delay of 0 minutes.

Chroma TOF 4.3X software of the LECO Corporation and the National Institute of Standards and Technology (NIST) database were used for measurement of raw peaks, data baseline filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak identification, and integration and spectrum match of the peak area.[28] Volatile compounds with less than 80% similarity compared with compounds in the NIST library and relative peak areas less than 0.1% were excluded from further statistical analyses. The relative peak area was calculated by dividing the peak area of each compound by that of the peak area of the IS in the same analytical run. The relative peak area of each category of compounds was the mean relative peak area of all compounds of each category. We ran a blank sample as a control to determine compounds that were derived from gland secretions of the bats and therefore considered to be endogenous. Compounds were assumed to be contaminants or exogenous compounds if they were in similar or higher concentrations in the blank sample than in the gland secretion sample. To avoid false positive compounds, only compounds detected in at least half of the samples from each individual were used for further analysis.

Behavioral experiment 1: individual odor discrimination

Forehead gland secretion collection. We collected 60 samples for the habituation phase of the behavior experiments (five samples each from 12 individuals; see below) and 12 samples for the discrimination phase of the behavioral experiments (one sample each from 12 individuals; see below). The 12 individuals were then tested in habituation–discrimination tests. As with samples used to test for individual variation, replicate samples for the habituation–discrimination tests were collected at intervals of 15 days. We were able to collect a full set of samples from each bat for this part of the experiment. After collection, we weighed the secretions to within $\pm$0.001 g (AR2140, Ohaus International Trading Co. Ltd, China). All samples were stored at –80 °C until used.

Habituation–discrimination tests. We used habituation–discrimination tests to determine whether the bats could distinguish individual

differences in the odor of the gland secretions.[29,30] In the habituation phase, the subject was presented with two swabs once a day for 4 days. One odor was from a single individual ("habituation odor") and the other odor was from an odorless swab. The odorless swab was used as a control to verify that the tested bats habituated to the odor instead of the swab. The bats spent a certain amount of time detecting the swabs when they were first presented. We considered the bats to be habituated to the odor if the duration of detection of the odorous swab was significantly longer than the duration of the odorless swab in the first habituation trial and if the duration of detection decreased significantly in each habituation trial. We also considered the bats to be habituated if the difference in duration of detection between the odorous swab and odorless swab diminished over the next three habituation trials.

The discrimination phase was conducted on the fifth day. This phase involved presenting the bat with two odors, one was the habituation odor and the second was an odor from another individual ("novel odor"). This phase was used to determine whether the tested subject could discriminate between the two odors. If the duration of detection of the novel odor for the tested subject was significantly longer than the habituation odor, we assumed that the tested subject could discriminate the two odors. The side on which each sample was placed was randomly selected by using the RAND function in Excel.

We performed all habituation–discrimination tests in a scentless plastic box (made of polymethyl methacrylate) without a plastic top (0.56 m long × 0.40 m wide × 0.32 m high; Fig. 1B) in a 4.5 m long × 2.4 m wide × 2.2 m high room. All tests were conducted between 19:00 and 22:00. The top of the box was covered with a wire mesh (0.76 m long × 0.60 m wide), which enabled the bats to hang from the roof. The two odor sources were presented through 1-cm diameter holes in the side of the box. The holes were placed 15 cm from the wire mesh, which was the average distance from a bat's head to toe. The distance between the two holes was also 15 centimeters. The wire mesh on the roof could be rotated so that the bats were facing directly toward and were equidistant from the position of the odor sources.

For the habituation phase, a bat was released into the center of the experimental set-up and given at least 5 min until it calmed down (i.e., remained motionless), at which point the two swabs were presented. Before the trial, we placed vials containing the gland secretions on ice until the secretions thawed completely (usually 10–20 min). After the bat became motionless, we placed 5 mg of gland secretions on a swab (20 cm in length and 2.8 mm in diameter) and inserted two swabs into two pieces of foam to stabilize them. We then moved both swabs slowly and simultaneously toward the bat at a constant rate. The distance between each swab and the bat was 10 cm (Fig. 1C), mimicking natural conditions. Considering the rapid volatilization of volatile compounds in gland secretions, the duration of each experiment was 10 minutes. We recorded the behavior of the bat for 10 min via an infrared camera (FDR-AX60; Sony Corp., Tokyo, Japan), which was placed 0.3 m in front of the box. Odor detection was defined as the bat moving its head toward the swab up to a distance of $\leq$1 cm and spending at least 1 s on it at a time (Video S2, online only). The duration of detection was defined as the total time spent interacting with the odor. Each odor sample was used only once. If a swab was licked or dislodged, the data collected from that pair of swabs were not included in the data set. After each trial, we cleaned the plastic box using 75% ethyl alcohol to remove volatile compounds, and we ventilated the room by opening a set of windows. The time interval between two consecutive trials was at least 10 minutes.

Behavioral experiment 2: manipulation of forehead gland protrusion during agonistic encounters

Animals and housing in the field. To test the function of chemical signals in conflict resolution, we caught 120 adult males from Yunnan (96 males) and Guizhou (24 males) in July–August 2019. We captured at most 16 adult males at a time, and captured bats every 1 or 2 days. Captured bats were housed for at least 24 h in individual cages (0.5 m long × 0.5 m wide × 0.5 m high) in a makeshift laboratory in the field (6.0 m long × 3.4 m wide × 2.9 m high) before the experiment started. The room was maintained at a relative humidity of around 65% and a temperature between 20 and 25 °C, as recorded by a hygrothermograph (YHZ-90450, Meiliju Ltd., Shenzhen, China). Bats were given *ad libitum* freshwater and larvae of *Z. morio* enriched with vitamins and minerals. Each bat was used only once.

Morphological measurements. The body mass of each individual was measured using an electronic balance (±0.01 g; DH-I2000, Diheng Ltd., Shenzhen, China). We measured the length of the right forearm of each individual using an electronic vernier caliper (±0.01 mm; 111-101V-10G, Guanglu Ltd., Shenzhen, China) before the trials. We measured body mass and forearm length of each individual three times, and their averages were used for further analysis.

Staged territorial agonistic interactions. We conducted agonistic interactions between pairs of male *H. armiger* in a box made of acrylic sheet plexiglass without a lid (1.00 m long × 0.50 m wide × 0.50 m high; Fig. 1D). The box was placed on two benches 0.35 m above the ground in a second temporary laboratory (6.00 m long × 3.40 m wide × 2.90 m high). The room was closed during the experiments, thereby eliminating any effect of airflow on the bats' response to odors. The temperature of the room was 20–25 °C and the humidity was 50–70%. We placed two infrared high-speed cameras (Photonfocus MV1-D1312IE-240-CL-8) in two front windows to record the bats' behavior and to detect the presence of gland protrusion. The sampling rate was 85 frames/second. Data were extracted from the video using a rate of 25 frames/second (Video S1, online only). We also set up an infrared spotlight (KTJ-GY-300W-42V; RockeTech Corp., Ltd., Hunan, China), which was mounted 1.20 m away from the box and 1.50 m above the ground, to provide sufficient illumination for the infrared high-speed cameras. We placed two infrared cameras (FDR-AX60; Sony Corp., Tokyo, Japan) in two back windows to record the duration of any aggressive interaction. The two infrared high-speed cameras and two infrared cameras were mounted 0.55 m above the ground. We used an Avisoft UltraSoundGate 116H (Avisoft Bioacoustics, Glienicke, Germany) with an ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics) at 0.55 m from the right window of the box to record the bats' vocalizations. The sampling frequency was at 250 kHz with 16-bit resolution. Pairs of males were selected randomly and placed individually in the center of the two pieces of wire mesh (0.36 m long × 0.25 m wide) on opposite ends of a slide rail (2.00 m long). Each piece of wire mesh was fixed by four pulleys to slide steadily on these two rails.

We removed the hair around the forehead gland before the trial to clearly detect the presence of a protrusion from the forehead gland and to avoid recapturing the tested bats. To minimize the effect of body size on contest duration, intensity, and outcome, we staged dyadic agonistic interactions between the paired males matched in body mass (the ratio of body mass between males was between 0.9 and 1.1[23]) and in forearm length (the difference between males in forearm length/male-average length $<2 \pm 1.2\%$[31]). We carried out trials where both individuals had functioning glands, neither had a functioning gland, or only one had a functioning gland. We disabled gland protrusion by gluing the facial cuticle over the glandular region, thereby completely preventing the forehead gland from protruding during the experiment. Temporarily sealing the gland opening could guarantee that the scent was absent because the bats do not wipe the gland secretions on their fur or anywhere over their body (C.Z., personal observation). We waited at least 5 min before starting the experiment. The adhesive had solidified before the onset of the experiment, so it generated no volatile odor. We used 60 pairs of males (20 pairs/group) in this experiment. The glue (502, deli) was easily removed after the experiment and had no negative effects on bats (C.Z., personal observation). The bats that had temporarily disabled gland protrusions were not tested for their agonistic behavior again after the glue was removed because fighting experience from previous interactions can affect the outcome of subsequent contests.

Before the trial, we randomly selected two males matched in body size and placed them in the center of the two pieces of wire mesh to allow them to acclimate to the box. After the two males remained motionless for at least 5 min (i.e., no body movements), C.S. manually pulled the two pieces of wire mesh (approximately 0.5 m apart) attached to four pulleys slowly and simultaneously at a constant rate (about 2.5 cm/s) toward each other by a rope until they reached the center of the box; this simulated two bats invading each other's territory. The distance between the two bats was approximately 15 centimeters. The process of pulling the wire mesh toward each other occasionally disturbed one of the bats causing it to fly off the mesh. We discarded such unsuccessful trials. The tested bats from unsuccessful trials were returned to the individual cages and were used in subsequent attempts.

We assumed that an agonistic behavior pattern shown during the staged interactions began when one of the opponents started the first wing flapping bout or boxing. Following Sun *et al.*,[23] agonistic interactions were terminated when we could determine a clear winner and loser within 15 min of the start of the trial. The trial was terminated if a winner could not be determined within the 15-min interval. We defined the winner as the individual that remained in place after the loser had retreated. We defined the loser as the individual that retreated after an agonistic interaction and failed to display any aggressive behaviors after retreat for at least 20 seconds. The experiments were performed during the maximal agonistic interaction activity period (between 20:00 and 08:00). After the trial, we cleaned the box using 75% ethyl alcohol to exclude the potential effect of odor on the next trial, and the room was well-ventilated to remove odor residues.

Behavioral analysis

We used QvodPlayer (Version 5.0.80, Shenzhen Qvod Technology Co., Ltd., Guangdong, China) to analyze video recordings from 60 dyadic agonistic interactions and to measure agonistic behavior. To compare the differences in contest duration and intensity among the three paired-male treatments, we analyzed contest duration and the proportion of interactions resulting in a physical fight (Table S1, online only).

An experimental blinded method was conducted to reduce observer bias for the habituation–discrimination test and for the agonistic interactions test. Here, H.G. was the blinded observer.

Statistical analysis

We calculated the relative peak area of the GC-MS peaks using the peak area of each compound divided by the peak area of the IS. On the basis of the relative peak area, we calculated the Bray–Curtis similarity index between each pair of samples, and then used this to perform a nonmetric multidimensional scaling (NMDS) ordination. This placed each sample in a two-dimensional space so that the relative distance between the samples matched their chemical similarity. "Stress" was used to measure goodness of fit, which assessed how well a particular configuration recreated the observed distance matrix associated with the data. We used the following criteria for stress results: stress <0.05 showed an excellent representation in two dimensions; $0.05 <$ stress <0.1 was very good; $0.1 <$ stress <0.2 was good, and stress >0.2 showed a poor representation in two dimensions.[32]

In this study, we determined the chemical composition of gland secretions from seven individuals that contributed at least three samples. We computed a nonparametric analysis of similarities (ANOSIM) with 1000 permutations on the basis of the Bray–Curtis similarity distance matrix of each individual. The ANOSIM test is a series of Mantel-type permutation or randomization procedures that do not require any assumptions about the distribution of the data.[33] We defined global R as the difference in average rank dissimilarity within individuals compared with between individuals. The closer R is to 1, the more the samples from the same individual are similar to each other, and the more different they are to samples from other individuals.[33]

To compare the difference in the relative peak area of major categories of compounds among the seven individuals, we computed a Bray–Curtis similarity index using the average relative peak area of each category of compounds in the ANOSIM test procedure with 1000 permutations. To examine whether the chemical composition of each individual changed over time (i.e., from day 0 to day 45 to day 90), we compared the chemical composition of gland secretions from seven individuals at the three sampling dates using the ANOSIM test procedure with 1000 permutations. The NMDS plot was based on Bray–Curtis similarities.

To examine whether the males responded differently toward forehead gland odors from different individuals, we first used Kolmogorov–Smirnov tests to test the normality of duration of detection ($P > 0.05$). For the habituation phase, a one-way repeated-measures analysis of variance (ANOVA) was used to test differences in the duration of detection when the tested bats were repeatedly presented with the habituation odor over 4 days. Mauchly's test indicated that the sphericity assumption was violated for the duration of detection ($\chi^2 = 29.833$, $P < 0.0001$). Thus, a Greenhouse–Geisser correction was used to adjust the degrees of freedom ($\varepsilon = 0.549$ for the duration of detection). Furthermore, we used Bonferroni post hoc tests to test the differences in the duration of detection across days. Moreover, paired-sample t-tests were used to test differences in the duration of detection between the odorous and odorless swabs in all habituation trials.

For the discrimination phase, paired-sample *t*-tests were used to compare differences in the durations of detection between the habituation and novel odors.

All individuals used in trials where both bats were capable of gland protrusion and in trials where both bats were incapable of gland protrusion were collected from caves in Yunnan Province. Mixed pairs (i.e., only one individual had a functional gland) were derived from two source populations. Sixteen adult males used in the mixed-pair trials were caught in Yunnan Province and 24 adult males were caught in Guizhou Province. The bats from Guizhou Province were not included in either the control (i.e., both with a functional gland) or experimental (i.e., neither with a functional gland) groups because we used bats from Yunnan Province first instead of bats from Guizhou Province to complete the two trials (i.e., control and experimental groups). In the mixed-pair trials, we found that there were not enough bats to carry out these trials, we thus introduced some bats from Guizhou Province to complete the remaining trials. An independent sample *t*-test was used to determine the impact of population on contest duration between bats from Yunnan and Guizhou Provinces.

Some bats vocalized during the staged contests. One-way ANOVA was used to test the effects of this vocal behavior on contest duration. In all cases, vocal behavior was treated as present or absent for each member of the pair. Thus, vocal behavior was either (1) vocalizations from both contestants, (2) from only one contestant, or (3) from neither contestant.

To test the potential function of the chemical signals in conflict solution, we first used a $\log_{10}$-transformation to normalize the contest duration. A one-way ANOVA was used to compare the difference in the contest duration among the three treatments of paired bats, and a Tukey's multiple-comparison test was performed if a significant difference was found. Pearson's chi-square tests were used to assess whether contestants in trials where neither bat had functioning glands tended to be involved in contests with more physical contact than contests where at least one bat had a functioning gland. We used exact binomial probability tests to estimate whether contests tended to be won by the male that was capable of gland protrusion when the other individual's gland was glued shut.

Table 1. Major categories of compounds identified from the forehead gland of seven bats

Number	Category of compounds	Proportion in this study (%)
1	Alkane	19
2	Alcohol	14
3	Aldehyde	14
4	Ketone	11
5	Carboxylic acid	10
6	Others	32

Statistical analyses of chemical data were performed using the VEGAN package[34] in R (v. 3.6.3).[35] Statistical analyses of behavioral data were performed with SPSS® 22.0 (IBM Corp., Armonk, NY). We considered $P < 0.05$ as significant.

Data availability

The data used in this study have been archived on the Dryad Digital Repository and are available at https://doi.org/10.5061/dryad.8931zcrrp.

Results

Scent profiles

We detected a total of 431 volatile compounds in 33 samples from seven individuals (mean ± SE = 8.76 ± 0.30 mg/sample; range: 5–13 mg). A total of 84 volatile compounds remained after filtering and these compounds were used for further statistical analyses (Table S2, online only). Detected volatile compounds were classified into the following 16 categories: alkane, alcohol, aldehyde, ketone, carboxylic acid, ester, phenyl, ether, terpene, phenol, alkene, amine, peroxide, ammonium salt, nitrile, and sulfone. Among them, the first five categories accounted for 68% of the 84 compounds (Table 1).

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 $R = 0.183$, $P = 0.008$; Fig. 2A). On the basis of the average of the relative peak area of each category of compounds, there were also significant differences in categories of compounds between different individuals (ANOSIM: global $R = 0.196$, $P = 0.004$; Fig. 2B). Individually specific compounds in samples are shown in Table 2.

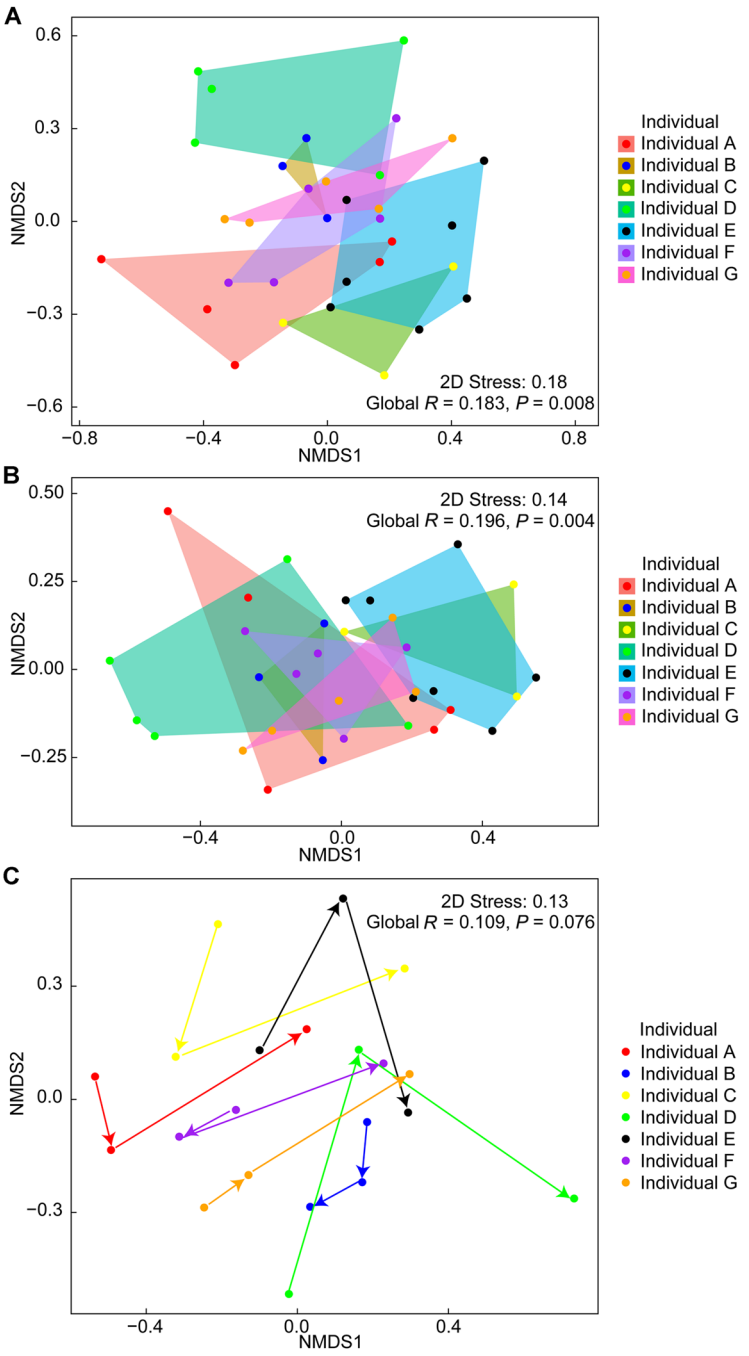

Figure 2. Nonmetric multidimensional scaling plots showing (A) the similarity in chemical composition of 33 samples from seven individuals, (B) chemical similarity of major categories of compounds identified from the forehead gland of seven bats, and (C) changes in the scent profiles of seven individuals over a period of 3 months. Arrows indicate the sequence of sampling from the first to the last. Each color represents one individual. Nearby samples have a similar scent and distant samples have a dissimilar scent. Axes are dimensionless. In panel C, the changes in the chemical profile of each individual can be plotted on the same scale because the approach (i.e., the Bray–Curtis similarity index and NMDS; see Materials and methods section for details) takes into account changes in the chemical composition with each individual and the degree of variation between different individuals.

Table 2. Description of the number of compounds in individual *Hipposideros armiger* gland secretion with a minimum of three repeat samples

Individual	Mean number of compounds per individual ± SE	Number of compounds occurring in all samples from the individual	Individually specific compounds in samples	*n*
Individual A	65 ± 2.4	38	4	5
Individual B	65 ± 1.5	50	0	3
Individual C	66 ± 1.2	49	2	3
Individual D	59 ± 3.4	30	0	5
Individual E	66 ± 1.3	39	1	7
Individual F	64 ± 2.9	41	1	5
Individual G	60 ± 3.2	38	0	5

NOTE: *n* indicates the number of samples from each individual.

Individual A had four specific compounds that were not detected in other individuals; individual C had two specific compounds; individuals E and F had one specific compound each. Additionally, for each individual, the chemical composition of the glandular secretions did not change significantly over time (ANOSIM: global $R = 0.109$, $P = 0.08$; Fig. 2C).

Individual discrimination

We tested 12 males for their ability to discriminate the odors of glandular secretions from different individuals in the habituation–dishabituation tests. In the habituation phase, there were significant differences in the duration of detection for the tested bats across the four habituation trials (one-way repeated-measures ANOVA: $F(1.646,18.107) = 25.444$, $P < 0.005$). Bonferroni post hoc tests showed that the duration of detection decreased significantly from the first presentation to the subsequent three presentations ($P = 0.033$–0.0002; Fig. 3). Moreover, males detected the scented swabs significantly longer than the unscented swabs during the first habituation trial ($t_{11} = 3.795$, $P = 0.003$; Fig. 3), while they did not detect the swabs differently during the next three habituation trials ($t_{11} = 0.719$–1.605, $P = 0.137$–0.487; Fig. 3). These results indicate habituation to the odors. In the discrimination phase, the duration of detection of the novel odor was significantly longer than the duration of detection of the habituation odor (paired *t*-test: $t_{11} = 8.131$, $P < 0.001$; Fig. 3), which indicated a dishabituation response. These results suggest that bats could discriminate individual differences via an odor from the forehead gland secretions.

Effects of intact forehead gland on agonistic interactions

No significant differences were found in the contest duration between the Yunnan population and the Guizhou population (independent sample *t*-test: $t_{18} = -1.148$, $P = 0.266$). Therefore, we removed the effects of population on contest duration.

There were no significant differences in contest duration (ANOVA: $F(2,17) = 1.946$–2.906, $P = 0.082$–0.276; Fig. S2, online only) between the three vocalization categories for any of the pair-type treatments. Therefore, we removed the effects of vocalization from the model of contest duration.

There were significant differences in the proportion of physical contact among the three pair-type treatments (Pearson's chi-square test: $\chi^2 = 6.933$, d.f. = 2, $P = 0.031$; Fig. 4A). However, there were no significant differences in the proportion of physical contact between trials with intact-gland males versus mixed pairs (Pearson's chi-square test: $\chi^2 = 2.849$, d.f. = 1, $P = 0.091$; Fig. 4A) or between mixed pairs and pairs where both bats had no gland protrusion (Pearson's chi-square test: $\chi^2 = 0.784$, d.f. = 1, $P = 0.376$; Fig. 4A). In contrast, there was a significant difference in the proportion of physical contact between intact-gland pairs and pairs where neither bat had a functioning gland (Pearson's chi-square test: $\chi^2 = 6.144$, d.f. = 1, $P = 0.013$; Fig. 4A).

There were significant differences in contest duration among the three pair-type treatments (ANOVA: $F(2,57) = 4.64$, $P = 0.014$; Fig. 4B). The Tukey's multiple-comparison test showed that the only significant difference in pair-type treatments was observed between pairs with functioning glands and pairs without functioning glands (Fig. 4B).

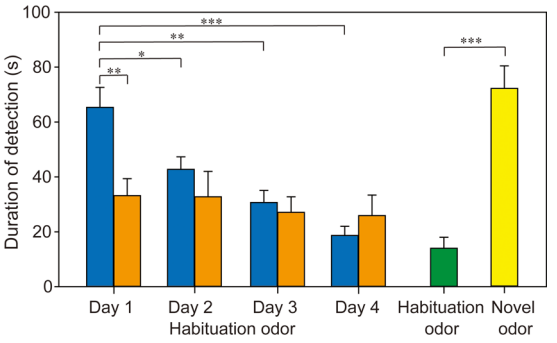

Figure 3. **Duration of detection of gland odors by *Hipposideros armiger* in the habituation–discrimination tests. Blue columns represent habituation trials with the scented swab; orange columns represent habituation trials with the unscented swab; green column represents discrimination trials with habituation odor; yellow column represents discrimination trials with the novel odor. Data are shown as means ± standard errors. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.**

In interactions between mixed pairs, we could determine a clear winner and loser in 18 of the 20 interactions. Among the 18 interactions, 16 (89%) were won by the individuals with a functioning gland, and 2 (11%) were won by the individuals without a functioning gland. Thus, individuals with a functioning gland were significantly more likely to win during agonistic interactions compared with individuals without a functioning gland (binomial test: $P = 0.001$; Fig. 4C).

Discussion

In this study, we found that there were significant differences in the concentrations and categories of compounds between individuals, and bats could use chemical signals for individual recognition, supporting our first hypothesis. Additionally, there were significant increases in the proportion of physical contact and contest duration when gland protrusion was prevented in both opponents, which supported our first prediction of the second hypothesis. Moreover, we found that males with gland protrusion won more contests than those without gland protrusion, supporting the second prediction of the second hypothesis.

Individual odor signatures

High-resolution GC-MS instruments are a powerful tool to determine individual differences in chemical compounds. Here, significant differences in the concentrations and categories of chemical compounds were observed among individuals, and different individuals had individual-specific compounds. These results suggest that the odors of forehead gland secretions of *H. armiger* potentially convey individual identity information. Similar results have been found in the Bechstein's bat (*Myotis bechsteinii*),[16] greater spear-nosed bats (*Phyllostomus hastatus*),[19] and male greater sac-winged bats (*Saccopteryx bilineata*).[17] These results suggest that individual differences in chemical signals in bats are common, which provides a basis for individual recognition in a large and complex social system with multiple species forming large colonies and with diverse mating systems.

Individual recognition via chemical signals

Selective pressures drive social animals to clearly signal their individual identity to others, which is important but difficult in a large and complex social system.[36] Given the incredible information content of chemical signals, olfaction appears to be an ideal sensory modality for conveying individual identity as well as multiple other types of information.[37] Elements of chemical signals can also have functions other than information content. Here, we found that alkane, alcohol, and carboxylic acid accounted for 43% of the chemical signals of *H. armiger*. In addition to functioning in recognition and range marking, prior studies showed that these chemical compounds could increase chemical stability and durability due to their slow degradation and resistance to degradation in water and heat.[1,38]

Furthermore, our behavioral recognition assays confirmed that *H. armiger* could discriminate the odors of forehead gland secretions from different individuals. Similar results have been found in Antarctic seabirds (*Pachiptila desolata*),[39] mice (*Mus domesticus*),[40] and Belding's ground squirrels (*Spermophilus beldingi*).[41] These findings suggest that individual recognition via chemical signals in vertebrates is widespread. *H. armiger* has a polygynous mating system that includes a harem with one territorial male and several females.[42] Thus, selection on clearly signaling a territory owner's identity is especially crucial in male *H. armiger*. Because harem males defend their females or defend a territory against other males, the ability of harem males to distinguish between neighbors and strangers based on chemical signals could mitigate energy expenditure by reducing the number of agonistic

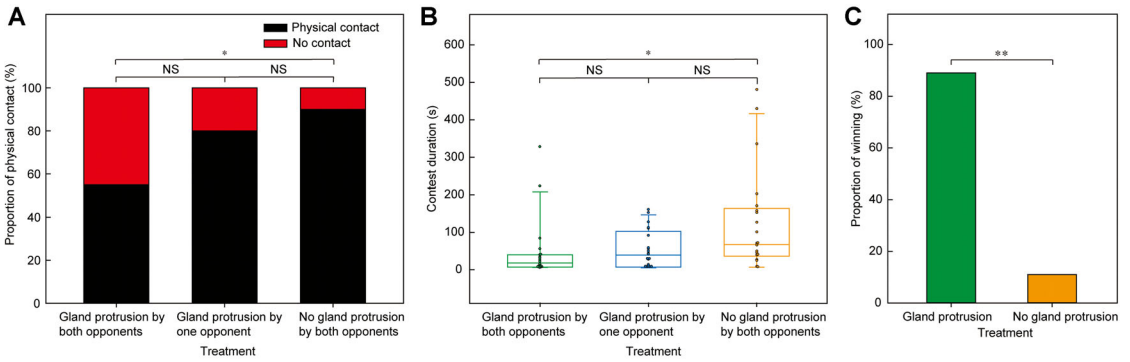

Figure 4. **Effects of gland manipulation trials. (A) The proportion of physical contact in different treatments. (B) The contest duration in different treatments. (C) The proportion of winning in a mixed pair (i.e., only one individual with odor). "No contact" means there is no physical contact between opponents during an aggressive interaction. *$P < 0.05$; **$P < 0.01$; NS indicate no significant difference ($P > 0.05$).**

interactions between males or reducing the escalation of agonistic interactions to physical combat. For example, individual recognition would allow harem males to recognize the status of other males and only remain highly aggressive toward non-harem males that are likely to pose more of a threat than harem males.[43] Our results also indicate that integration of chemical composition analysis and behavioral assays is helpful to fully understand roles of chemical signals in intraspecific communication.

Chemical signals for conflict resolution in territory defense

A key question in conflict resolution is what is the fitness benefit of using communication signals in agonistic interactions? A previous study showed that the frequency of fighting and contest duration significantly increased after chemical signals were prevented in both contestants in cave crickets (*Troglophilus neglectus*), suggesting that chemical signals can reduce the costs of a fight.[44] In our study, we found that interactions between males that could not protrude their glands resulted in a higher proportion of physical contact and longer contest duration than interactions between males that were capable of protruding their glands. This fact is important because it shows that preventing gland protrusion may not affect males' vigor, and that winning intact males in mixed pairs did not win simply due to reduced vigor of their opponent caused by gland manipulation. More generally, these results suggest that chemical signals in *H. armiger* can reduce fight costs because they can function in territorial conflict resolution. On the basis of these results, we can infer that chemical signals in *H. armiger* may advertise the resource holding potential (RHP) of signalers; RHP normally correlates with traits such as body size, strength, physiological condition, and endurance.[45]

However, we should be cautious of the role of odor in conflict resolution because the process of altering the gland by itself may have contributed to our results in the tests on conflict resolution. For example, altered bats may be more prone to losing matches because of the alteration itself, which may have created additional stress. Unfortunately, it is difficult to do a simulated alteration where unaltered bats go through a similar process as the altered ones. An interesting treatment that could be done during a follow-up study would be to paint gland exudate onto a nonmanipulated bat. One problem with this treatment is that it would be difficult to ensure that the level of volatile components is the same as those emanating from an intact gland. This is important because the volatile compounds probably carry most of the olfactory information, but they volatize rapidly. As such, this treatment would need to be run in addition to the experiments we describe here.

The potential for volatility in the gland exudate is underscored by the fact that we found that aldehyde and ketone accounted for 25% in the forehead gland secretions of *H. armiger*. These chemical compounds with carbonyl functional groups are more soluble in water than those with carboxyl or hydroxyl functional groups. This characteristic

promotes rapid volatilization and potentially serves a function of threat,[1,38] suggesting chemical signals in *H. armiger* may contain threat information to advertise fight ability. Thus, we suggest that chemical signals of *H. armiger* can mitigate the costs of conflict during territory defense because the level of aggression and contest duration are strongly associated with the energetic cost of conflict, injury, or risk of predation.[46,47] However, further behavioral experiments are needed to determine which compound categories may function as threat signals and to elucidate causal links between chemical signals and fighting ability in *H. armiger*.

Social vocalization and agonistic interactions

Acoustic communication can function in reducing the cost of agonistic interactions.[6] Acoustic signals can also convey information about individual identity and aggressive motivation, which can make contestants decide whether to continue or cease fighting.[1] Similar results have been obtained in male Seba's short-tailed fruit bats (*Carollia perspicillata*),[25] Asian particolored bats (*Vespertilio sinensis*),[48] and Indian false vampire bats (*Megaderma lyra*).[49] *H. armiger* use agonistic displays to defend their roosting territory, accompanied by vocal signals.[23] In this study, there were no significant differences in the contest duration between the three vocalization categories for each pair condition, suggesting that acoustic signals may not substantially affect contest duration under conditions such as those used in this study. Vocalizations normally honestly encode body size information of senders.[50,51] In this study, agonistic interactions were designed to occur between size-matched opponents, which may limit the relevance of vocalizations to contest duration. Together, our results suggest that social vocalization of *H. armiger* may not significantly affect agonistic interactions between size-matched opponents for territory conflict. However, additional multimodal playback experiments including acoustic and chemical signals are needed to evaluate the relative importance of vocalizations and odors in conflict resolution of *H. armiger* during territory defense.

Conclusion

In summary, our study demonstrated that the chemical signals from forehead gland secretions of male *H. armiger* varied among individuals for recognition, and can help to resolve territorial conflict. Therefore, our study provides evidence that chemical communication can play a pivotal role in conflict resolution in mammals. Our results also confirmed that integration of chemical composition analysis and behavioral assays provides a powerful tool to fully understand the role of chemical signals in animal communication. The limitation of this study is that it is difficult to determine if the chemical signals of *H. armiger* are associated with the potential indices of RHP, such as strength, physiological condition, and endurance, based on our present data. Further studies will need to determine which compounds play a part in individual recognition and conflict resolution, and determine the causal links between chemical signals and the potential indices of fighting ability.

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Author contributions

C.Z., C.S., and T.J. designed the study. C.Z., C. S., and H.G. collected the data. C.Z. and C.S. analyzed the data and wrote the manuscript. T.J., J.F., and J.R.L. revised the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Description of contest duration and behavioral parameters of male *H. armiger*.

Table S2. The ratios of the peak areas of each compound in each sample (peak area of each compound divided by the area of the internal standard).

Figure S1. An example of spectrogram of a bent-upward frequency modulation (bUFM) syllable territorial call of *H. armiger.*

Figure S2. Effect of vocalization on contest duration among three pair types.

Video S1. Video of male *H. armiger* during agonistic interactions with gland protrusion manipula-

tion. Filmed with two infrared high-speed cameras at 85 frames/s and played back at 25 frames/s.

Video S2. Video of *H. armiger* detecting two odors in the habituation–discrimination tests.

Competing interests

The authors declare no competing interests.

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