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Male Scent Gland Signals Mating Status in Greater Spear-Nosed Bats, *Phyllostomus hastatus*

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Abstract

Chemical signals are ubiquitous, but often overlooked as potentially important for conveying information relevant for sexual selection. The male greater spear-nosed bat, *Phyllostomus hastatus*, possesses a sexually dimorphic gland on the chest that produces an odoriferous secretion. Here, we investigate the potential for this glandular secretion to act as a sexually selected olfactory signal by examining gland activity in and out of the mating season and determining if variation in its chemical composition reflects variation in male mating status or attributes of the individual. Based on gas chromatography-mass spectrometry (GC-MS) measurements of samples collected from wild bats roosting in caves in Trinidad, West Indies, we find that males that defend and roost with groups of females (harem holders) have significantly different chemical profiles from males found roosting in all male groups (bachelors). Additionally, profiles differed significantly among individuals. Taken together, these results suggest that this chemical signal has the potential to communicate both mating status and individual identity and thus could be used to mediate interactions among individuals within this harem-based social mating system.

Keywords Chemical communication · Olfaction · GC-MS · Sexual selection · Bats

Introduction

Sexual selection has produced elaborate signals for advertising competitiveness to rivals or quality to potential mates (Darwin 1871). Communication between rivals is important for mediating intrasexual competition by reducing direct conflict, particularly in species where strong male-male competition may cause injuries (Andersson 1994). In the presence of female choice, signals may reveal desirable features to potential mates, such as genetic quality or provisioning ability. While acoustic and visual signals are commonly studied modalities, increasing evidence indicates that olfactory signals can also play a role in sexual selection and provide information about competitiveness and mate quality (Johansson and Jones 2007; Martin et al. 2018; Penn and Potts 1998; Rich and Hurst 1998).

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Olfactory signals can communicate information in multiple ways, ranging from the rate and placement of signals to their chemical content. In territorial species, scent marking the perimeter of a territory is a common behavior. Because investment in odorant production and time spent depositing scent marks is costly (Gosling and Roberts 2001; Gosling et al. 2000; Harris et al. 2018; Radwan et al. 2006), the abundance of marks and the ability to countermark act as a signal of competitive ability to both rivals and potential mates (Fisher et al. 2003; Rich and Hurst 1998; Rich and Hurst 1999). Similarly, the height of a scent mark left on a vertical substrate by a terrestrial mammal can reveal the signaler's body size, which is often correlated with competitive ability (Sharpe 2015). Many mammalian olfactory signals are complex chemical blends, and variation in the presence/absence or relative abundance of constituents can reveal attributes of the signaler relevant to intra- and inter-sexual selection, including social status (Buesching et al. 2002a; Setchell et al. 2010), body condition (Buesching et al. 2002a; Buesching et al. 2002b; Ferkin et al. 1997), age (Caspers et al. 2011; Leclaire et al. 2014), parasite load (Kavaliers and Colwell 1995; Munoz-Romo and Kunz 2009; Penn and Potts 1998), immunocompetence (Rantala et al. 2002; Zala et al. 2004), and hormone levels (Burgener et al. 2009). The connection between these traits and the chemical profile is mediated by shared

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physiological and genetic mechanisms. For example, several studies report an association between chemical profiles and genetic diversity at major histocompatibility complex (MHC) loci (e.g. Lanyon et al. 2007; Radwan et al. 2008; Setchell et al. 2011). The MHC influences immune function in vertebrates, thus affecting the parasite load (Kurtz et al. 2004; Westerdahl et al. 2005) and microbial community (Bolnick et al. 2014; Kubinak et al. 2015), which can subsequently affect olfactory profiles (Archie and Theis 2011; Lanyon et al. 2007; Penn and Potts 1998). Through various, often unknown, physiological connections, odor can also indicate genetic diversity or heterozygosity at non-MHC loci (Leclaire et al. 2012; Overath et al. 2014; van Bergen et al. 2013), as well as genetic relatedness and compatibility (Charpentier et al. 2008; Charpentier et al. 2010; Penn 2002; Thomas and Simmons 2011).

Bats, the second most speciose order of mammals, possess a diversity of scent-producing glands on the face, around the genitals, and in the subaxillary region (Bloss 1999; Brooke and Decker 1996; Rehorek et al. 2010; Scully et al. 2000) and rely on chemical communication in multiple contexts (Bloss 1999; Dechmann and Safi 2005). Prior studies have demonstrated the utility of scent in the discrimination and recognition of colony members (Bloss et al. 2002; Bouchard 2001; Safi and Kerth 2003), individuals (Safi and Kerth 2003), and offspring (Gustin and McCracken 1987). However, with few exceptions (Caspers et al. 2008; Santos et al. 2016; e.g. Voigt and von Helversen 1999), the role of chemical communication in courtship and mating interactions is largely unknown among bats. The sexual dimorphism of the glands and scent-dispersing structures on many species (Bouchard 2001; Hickey and Fenton 1987; Scully et al. 2000; Tavares and Tejedor 2009), suggests that odor plays a role in intra- and intersexual interactions of many bats, which have diverse mating system types (McCracken and Wilkinson 2000).

In this study, we focus on the greater spear-nosed bat, *Phyllostomus hastatus*, in which adult males possess a large sebaceous gland on their chest that produces a thick white secretion with a pungent odor (Fig. 1). This gland is sexually dimorphic, as it is rudimentary and lacks secretory elements in females (James 1977). In addition to the glandular dimorphism, *P. hastatus* exhibits sexual size dimorphism (SSD), with males larger than females (McCracken and Bradbury 1981). Although this male-biased SSD is common among mammals (Weckerly 1998), it is atypical for bats, which often show reversed SSD due to the demands of flight during pregnancy and lactation (Ralls 1976). These dimorphic features are consistent with strong sexual selection in *P. hastatus*, which is further supported by their social behavior.

Like many other phyllostomid bats, *P. hastatus* exhibits female-defense harem polygyny, but the large size and long-term stability of the harem groups is relatively unique

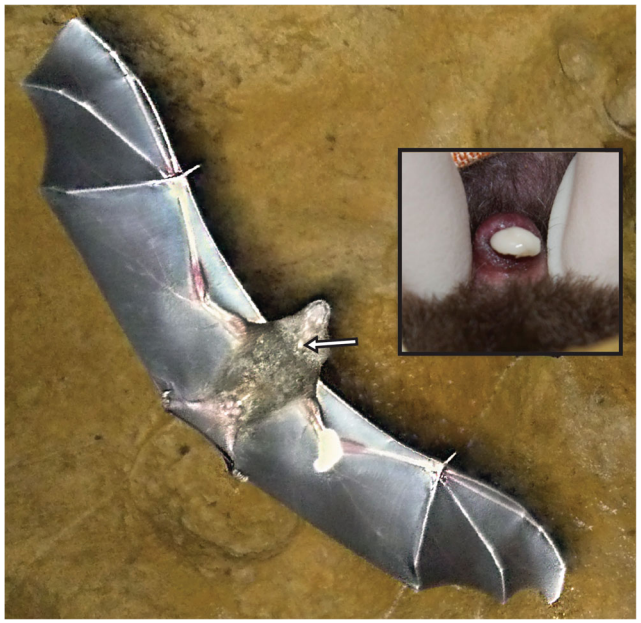

Fig. 1 Adult male *Phyllostomus hastatus* in flight (ventral surface). Arrow indicates the location of the chest gland, which is visible as a small bare patch. Inset shows close up of secretion being expressed from a chest gland by gloved fingers

(McCracken and Wilkinson 2000) and creates ample opportunity for sexual selection (Shuster and Wade 2003; Wade and Shuster 2004). Each harem group consists of 10–25 unrelated females (McCracken and Bradbury 1977; McCracken and Bradbury 1981) and is defended by a single male, who can retain tenure at a harem for up to 4 years (Wilkinson et al. 2016). Harem males smear the secretion from the chest gland onto the fur of the females within their harems, thus giving both males and females a pungent odor (McCracken and Bradbury 1981). Harem males attempt to monopolize matings, but the close proximity of neighboring harems and presence of large bachelor male groups may limit the degree to which harem males control paternity (McCracken and Bradbury 1977). Males in bachelor groups may roost together for many years and some may never attain harem status (Wilkinson et al. 2016). Given the priority access harem males have to mating opportunities, competition for access to females is intense. Evidence of fights is obvious in the many wounds and scars found on males' faces, bodies, and wings (personal observation).

Our aim is to determine if male chest gland secretions can communicate mating status, body condition, or individual identity. Males occasionally make forays into the harems of other males and the resident male drives away the intruders (McCracken and Bradbury 1981). Therefore, advertising status and physical attributes, such as body size and condition, may allow males to assess their opponents without escalation. By scent-marking the females, harem males may still be able communicate the risk of retaliation to intruders while absent from the harem. Currently, little is known about how males

acquire harems or who else sires offspring within harems, but the ability to advertise indicators of quality could potentially facilitate both of these events. Additionally, individuals in neighboring harems are likely to have repeated interactions due to the stability of roosting locations (McCracken and Bradbury 1981). Signals of individual identity would facilitate recognition of neighbors, which could mitigate potential conflict (Temeles 1994; Tibbetts and Dale 2007).

In this study we examine the composition of the glandular secretions via gas chromatography-mass spectrometry (GC-MS) and assess the potential information content of the signal by examining how variation in chemical profiles relates to variation in mating status (harem vs. bachelor), body size, body condition, and age of males. Using repeated samples taken from the same individuals over several days, we also evaluate the potential for the odor to reveal individual identity.

Methods and Materials

Study Population Bats were captured in three caves, Caura (10.7019°N, 61.3614°W), Guanapo (10.6942°N, 61.2654°W), and Tamana (10.4711°N, 61.1958°W) on the island of Trinidad, West Indies from December–January, 2012–2015. Each cave contained a colony of *P. hastatus* with up to 30 harem groups, some of which were previously banded (Wilkinson et al. 2016). Typically, each harem occupies a separate solution depression in the cave ceiling, but multiple groups may share large depressions and still remain spatially segregated. In Trinidad, *P. hastatus* exhibit a single breeding season from November to January, with most pups born in April (McCracken and Bradbury 1981; Porter and Wilkinson 2001).

We determined male mating status as either harem or bachelor by their roosting associations at the time of capture. We captured entire groups in the cave during the day (11:00–19:00) using a bucket extended on poles to the cave ceiling. A single adult male caught with a group of adult females was defined as a harem male. Males from groups containing multiple adult males, and occasionally non-reproductive females, were classified as bachelor males (McCracken and Bradbury 1981).

After capture, bats were held individually in cloth bags while each bat was processed. Previously banded bats were identified by their band number, and unbanded bats were fitted with a numbered metal band (Monel, National Band and Tag, Newport, KY, USA) on their forearm, with males banded on the right wing and females banded on the left. We recorded the mass (Pesola spring scale), forearm length (digital caliper; Chicago Brand, Medford, OR, USA), and degree of tooth wear (using a 5 category scale, cf. McCracken and Bradbury 1981) for each individual. Unless individuals were banded as newborn pups, tooth wear is currently the only way to estimate the age of living adult bats (Brunet-Rossinni and Wilkinson 2009). Male testes length and width were measured when possible, and testes volume was estimated assuming a prolate spheroid ($V = 4/3\pi r_w^2 r_l$, where r_w is half the width and r_l is half the length). The mating season for *P. hastatus* in Trinidad is from November to January (James 1977; McCracken and Bradbury 1981), and so testes were at or near their maximal size during the period of capture. To estimate body condition, we used the residuals from a linear regression of male body mass on forearm length from 154 adult males captured in the three caves. All measurements and samples used in these analyses were collected during the breeding season; however, additional observations of chest gland activity were made during the non-breeding season (April–June 2013, April 2018).

Sample Collection The male chest gland forms a deep pocket, which can be everted when palpated (Fig. 1). By gently squeezing the area around the gland, we extruded the white secretion and scooped it directly into a pre-cleaned glass vial with PTFE-lined septum. We wore a fresh pair of powder-free nitrile gloves for each sample to prevent contamination from our skin. After collection, the vials were immediately stored on ice until we returned to the field station where they were stored at −4 °C. To identify any potential contaminants, we collected one or more blanks on each sampling day in which we handled the vial exactly as during sample collection, but no sample was added to the vial. Samples and blanks were kept frozen during shipment to the US and then stored at to −80 °C until analysis.

In January 2015, we collected 50 samples from 31 individuals, including 20 bachelor and 11 harem males from Tamana Cave. Replicate samples were collected from males recaptured in this cave on subsequent days. Additionally, some males were brought back to the field station (William Beebe Tropical Research Station, Trinidad, West Indies, 10.69253°N, 61.28956°W) and held in individual cages for up to 6 days for behavioral testing, during which time additional samples were collected every 2 days. We were able to collect two or more replicate samples from 3 bachelor and 11 harem males.

GC-MS Analysis We isolated the non-polar and weakly polar compounds via an ether-water extraction, using 99.9% extra-pure methyl *tert*-butyl ether (MTBE, Acros Organics) and chromatography-grade water (Fisher Scientific). All glassware was doubly rinsed with MTBE prior to use. For the extraction, we added 500 μL of MTBE and 500 μL of water to each sample. Samples were then vortexed for 45 sec and centrifuged for 5 min at 3000 rpm. The MTBE supernatant was transferred to a new solvent-rinsed tube and stored on ice. An additional 500 μL of MTBE was added to the aqueous phase, mixed, centrifuged, and subsequently the MTBE phase

was pooled with the previous extraction on ice. This process was repeated for a third round, resulting in approximately 1.5 mL of MTBE extract. Each sample was concentrated to dryness on ice under a stream of ultra-high purity nitrogen, and the dried product was redissolved in 100 μL of MTBE before GC-MS measurements.

Samples were loaded into vials with solvent-rinsed glass chromatography inserts and stored at −80 °C until analysis. Just prior to analysis, an internal standard of 2.5 μL of hexachlorobenzene solution (2 mg HCB/mL MTBE) was added to each sample. To prevent extended delays between extraction and GC-MS analysis, the samples were processed in 3 batches, and all samples were analyzed within 36 hr of extraction.

The gas chromatography measurements were performed on an Agilent 6890 N system coupled with a JEOL high-resolution magnetic sector mass spectrometer (JMS-700 MStation) with the EI ion source (70 eV). The mass spectrometer was operated in the mode of high scan speed and low resolution (1000) with the mass range from 50 to 600 Da. A silica capillary column (Agilent HP-5MS, 30 m length, 250 μm I.D.) was used with helium (at 1 ml/min) as the carrier gas. Analysis was performed as follows: injection volume was 1 μl, the inlet temperature was 280 °C in splitless mode, the column temperature was programmed from 50 °C at 1.0 min, then increased to 310 °C at the rate of 16 °C/min and then held at 310 °C for another 2.75 min.

All chromatographic data pre-processing was done using MALDIquant for R (Gibb and Strimmer 2012). Although this package is designed for MALDI-TOF data, many of the pre-processing functions are also appropriate for chromatographic data. First, we corrected for baseline shift using the SNIP algorithm. Because of slight variations in elution times between samples, chromatograms were aligned first by the internal standard and then further refined using a peak-based method, which employs a LOWESS warping function, utilizing a preliminary peak list (5-point half-window and a signal-to-noise ratio of 2). After alignment, peaks were automatically detected in all samples using a half-window of 2 and SNR of 0.5. We then used MStation software (JEOL, USA) to obtain the mass spectra, which we used to ensure the aligned peaks represent the same compounds. Peaks that show inconsistent spectra across samples were removed from subsequent statistical analyses. As a result, we retained only the subset of peaks that could be reliably matched and quantified across samples. By ignoring rare or low intensity compounds, differences among individuals or groups will be more conservative. To account for variation in total sample intensity, all analyses are based on relative abundance values, where the total abundance for each sample is defined as the sum of intensities for all retained peaks. Relative abundance proportions were transformed using the arcsine square-root prior to analysis. The sum of the raw intensities (total intensity) was recorded to account for variation in signal strength between samples.

Statistical Analysis All statistical analyses were performed in R (version 3.3.2, R Core Team 2016) via RStudio (RStudio Team 2015). We fit a generalized linear mixed model (GLMM) with a binomial error distribution and logit link function to test for effects of body size and condition on male mating status, including cave site as a random effect. Some males were captured and measured multiple times and so we calculated their average body size and condition. The significance of each predictor is evaluated via a likelihood ratio test (LRT) comparing a model with a term of interest to a model without that term. Because tooth wear is scored on a 5-point scale, we used a Fisher's exact test to assess differences between bachelor and harem males. We also compare the degree of testes development using a generalized linear model (GLM) with mating status and season as predictors of testes state (scrotal vs. abdominal) with a binomial error distribution. The significance of each variable is evaluated via LRT as above. We use a t-test to evaluate the difference in testes size between bachelor and harem males that have scrotal testes.

To evaluate the effect of male traits on the chemical profile, we used a permutational multivariate analysis of variance (PERMANOVA) with a Bray-Curtis distance matrix and 9999 permutations. To avoid negative eigenvalues, a constant was added to all non-diagonal dissimilarities (Legendre and Anderson 1999; Oksanen et al. 2017). We chose a Bray-Curtis distance because similarity is based only on compounds that are present in at least one sample, such that the absence of a compound in a pair of samples does not contribute to their similarity. This is especially important when zeros may be the result of detection ability, rather than true absence. Because PERMANOVA models lack AIC values, we used backward model selection, sequentially removing terms with the least explanatory power until all remaining terms were below an α_{crit} of 0.30. We chose a conservative α_{crit} to limit biases resulting from stepwise regression. When multiple terms had similar p-values ($p \pm 0.1$), we dropped each one alternately to examine their effects on the remaining terms before progressing. PERMANOVA is sensitive to differences in multivariate dispersion (Warton et al. 2012), so we also performed an analysis of multivariate homogeneity of group dispersions (betadisper function, vegan pacakage, Oksanen et al. 2017). Although we have replicate gland samples from some males, we included only the first sample collected from each individual in these analyses to remove potential effects of sample order or sample location (captive vs. wild). In the full model, we included male status, body size (forearm length), body condition, presence of scrotal testes, and age (tooth wear) as potential explanatory variables, as well as batch number, to account for potential variation between the three separate GC-MS runs, and the total signal intensity (sum of all raw peak intensities). To evaluate the effect of testes volume, we repeated the analyses using only the samples for which we had testes measurements.

To determine how well male mating status can be discriminated based on chemical profiles, we used a canonical analysis of principal coordinates (CAP: Anderson and Robinson 2003; Anderson and Willis 2003). This constrained ordination method, as implemented by the CAPdiscrim function in the BiodiversityR package (Kindt and Coe 2005), first performs a principal coordinate analysis (PCoA) followed by a linear discriminant analysis (LDA). We selected the number of PCoA axes (m) used in the LDA that provided the highest reclassification rate and significance was evaluated based on 999 permutations. Because this method can use only a single constraint, the effect of signal intensity is not accounted in this method. To further identify which compounds are associated with the status of each male, we performed an indicator species analysis (Dufrene and Legendre 1997) using the INDICSPECIES package (Caceres and Legendre 2009).

To evaluate the secretion's potential to signal individual identity, we used the same PERMANOVA and CAP methods as above; however, we included males with replicate samples. Factors included in the full model are individual identity nested within mating status, replicate number, batch number, and total intensity. To remove the effect of male mating status, the permutations are constrained by mating status.

Results

Mating Status, Morphology, and Age Bachelor and harem males do not differ in body size, as measured by forearm length (GLMM, $X^2 = 0.00$, $df = 1$, $p = 0.99$); however, they do differ in body condition (GLMM, $X^2 = 4.73$, $df = 1$, $p = 0.03$), such that harem males are heavier than bachelors given their skeletal body size. Additionally, harem males exhibit greater tooth wear than bachelor males (Median score: harem = 3, bachelor = 2; Fisher's exact, $N = 127$, $p < 0.001$), which suggests they tend to be older. The presence of scrotal testes is influenced by both mating status and season, such that harem males are more likely to have scrotal testes (GLM, $X^2 = 39.22$, $df = 1$, $p < 0.001$), and scrotal testes are more common in the breeding season (GLM, $X^2 = 35.03$, $df = 1$, $p < 0.001$). There is no significant interaction between season and mating status (GLM, $X^2 = 1.54$, $df = 1$, $p = 0.21$). When considering only the males with scrotal, and thus measurable testes, we find no significant difference in testes volume with respect to mating status ($t = 1.18$, $df = 90$, $p = 0.24$).

We observed that harem males maintain a continuously active chest gland, regardless of season ($N = 64$). Most bachelor males also possess an active chest year-round, but 13 of 122 bachelor observations showed an inactive chest gland during the breeding season. These bachelor males also had abdominal testes and tooth wear indicative of young age (score: 2), with one exception who had slightly greater tooth wear (score: 3).

Mating Status and Chemical Composition Automated peak detection detected 62–102 peaks per sample. However, after alignment and manual inspection, several peaks could not be aligned reliably across samples (i.e. the mass spectra were inconsistent) due to low intensity or the tendency for isomers to co-elute. Two peaks (retention times: 16.75 sec and 18.09 sec) were detected in the blanks run between samples and thus excluded. As a result, only 43 peaks from the GC-MS were retained for statistical analysis (Fig. 2, Table 1). Of these 43 peaks, 33 were detected in all samples, and the remainder were detected in at least 80% of the samples.

We found significant effects of male mating status and batch number on the chemical profile, but no significant effects of body size, condition, presence of scrotal testes, or tooth wear (PERMANOVA, Table 2). Similarly, the CAP discriminant analysis on the chemical profiles correctly classified 18 of 20 bachelors and eight of the 11 harem males, for an overall success rate of 84% ($m = 7$, $p = 0.02$). We found no significant difference in multivariate dispersion between each group (betadisp analysis, $F_{1,29} = 0.01$, $p = 0.97$), indicating there is as much variation in chemical profile among harem males as there is among bachelor males (Fig. 3). Other male attributes (body size, condition, presence of scrotal testes, and tooth wear) do not significantly affect the GC-MS chemical profile (Table 2). Using the subset of males with testes size measurements, we find that testes size is also not a significant

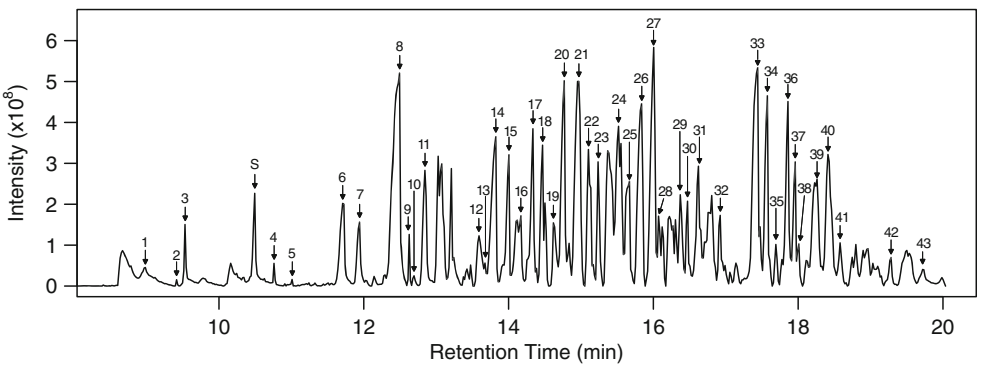

Fig. 2 Representative GC-MS chromatogram of male chest gland secretion. Numbered arrows indicate peaks used in statistical analyses. Peak labeled "S" denotes the internal standard

Table 1 Compounds detected via GC-MS and retained for statistical analyses

Peak No.	Retention time	HOS[a]	Relative abundance[b] (%)		Indicator[c]
			Harem	Bachelor	
1	8.96		0.64	0.52	
2	9.38	240	0.15	0.16	
3	9.49	210	1.18	1.18	
4	10.73	268	0.47	0.44	
5	10.98	268	0.18	0.24	
6	11.69	258	2.03	1.70	
7	11.94	272	1.78	1.76	
8	12.37	308	4.70	5.13	
9	12.59	308	1.36	1.78	
10	12.69	386	0.37	0.41	
11	12.81	285	2.50	2.04	H*
12	13.46	336	1.08	0.88	
13	13.64	336	1.16	1.46	
14	13.74	324	3.20	2.73	H*
15	13.96	386	2.34	2.25	
16	14.09	386	2.36	2.26	
17	14.29	362	3.20	2.53	H**
18	14.42	327	2.30	1.79	H**
19	14.61	327	1.09	1.01	
20	14.71	341	3.52	2.67	H***
21	14.92	341	4.42	4.45	
22	15.11	358	3.15	3.57	
23	15.24	341	2.28	1.65	H**
24	15.50	355	3.39	3.16	
25	15.62	325	2.62	2.33	
26	15.79	386	4.20	4.18	
27	15.97	367	4.92	5.32	
28	16.05	367	1.74	1.21	
29	16.35	383	2.27	2.39	
30	16.45	430	2.37	2.49	
31	16.59	414	2.84	3.35	B*
32	16.90	451	2.73	2.94	
33	17.37	508	4.82	4.96	
34	17.55	508	4.30	5.09	B***
35	17.69	465	1.50	2.39	B**
36	17.82	522	3.57	3.17	
37	17.92	479	2.47	2.37	
38	17.97	479	1.52	1.57	
39	18.20	493	2.23	1.95	
40	18.42	548	3.92	4.29	
41	18.60	548	1.82	2.54	B*
42	19.25	562	0.40	0.41	
43	19.68	576	0.94	1.26	

[a] Highest observed signal in the mass spectrum of the compound

[b] Abundance relative to the total abundance of the 43 peaks retained for statistical analysis

[c] H indicates positive association with harem males, B indicates positive association with bachelor males in indicator species analysis. Symbols indicate level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

predictor of the GC-MS profile ($F_{1,13} = 1.48$, $p = 0.20$). Indicator species analysis identified 10 compounds that show significantly different associations between the two male classes; six are more abundant in harem males and four are more abundant in bachelor males (Table 1, Fig. 4). Interestingly, all six of the harem male indicator compounds are apparently more volatile than the four bachelor male indicator compounds (Table 1). Such a sequence is highly nonrandom (Runs test, $Z = 2.67$, $p = 0.01$).

Table 2 Full and reduced models of male trait effects on chemical profiles

Effect	df	SS	pseudo-F	p
Full Model	10, 20	0.063	1.327	0.046
Status	1	0.007	1.511	0.104
Size	1	0.005	1.113	0.305
Condition	1	0.003	0.759	0.668
Age	3	0.012	0.897	0.653
Testes	1	0.006	1.393	0.148
Batch	2	0.016	1.709	0.034
Intensity	1	0.006	1.349	0.179
Reduced Model	6, 24	0.048	1.745	0.001
Status	1	0.009	2.034	0.033
Size	1	0.005	1.161	0.261
Condition	1	0.005	1.192	0.265
Batch	2	0.016	1.693	0.030
Intensity	1	0.007	1.417	0.127

Individual Identity and Chemical Composition The GC-MS profiles among individual males for which we have replicate samples ($N_{bach} = 3$, $N_{harem} = 9$) differed significantly independent of the effect of status (Table 3). We also found a significant effect of the batch, but not replicate. The CAP discriminant analysis, which is unable to account for the batch effect, was able to successfully classify 18 of the 29 samples (62.1%) to the correct individual ($m = 5$, $p > 0.05$).

Discussion

Adult male greater spear-nosed bats maintain an active chest gland throughout the year, regardless of their mating status. The chemical composition of the gland's secretion, however, differs significantly between bachelor and harem males. Based on our analysis of 43 compounds, we found that samples could be assigned to the correct mating status for 84% of individuals tested. This chemical signal thus has the potential to communicate male mating status to both rivals and potential mates. Ten compounds show significant differences between mating statuses; of these, harem male secretions contain greater relative abundance of the earlier eluting (smaller, lighter) compounds, while those of bachelor males exhibit greater abundance of the later eluting (larger, heavier) compounds. This non-random pattern may reflect differences in volatility; however, further analysis of the chemical structures will be needed.

Although we did not find any significant association between the chemical profile and other male attributes, such as

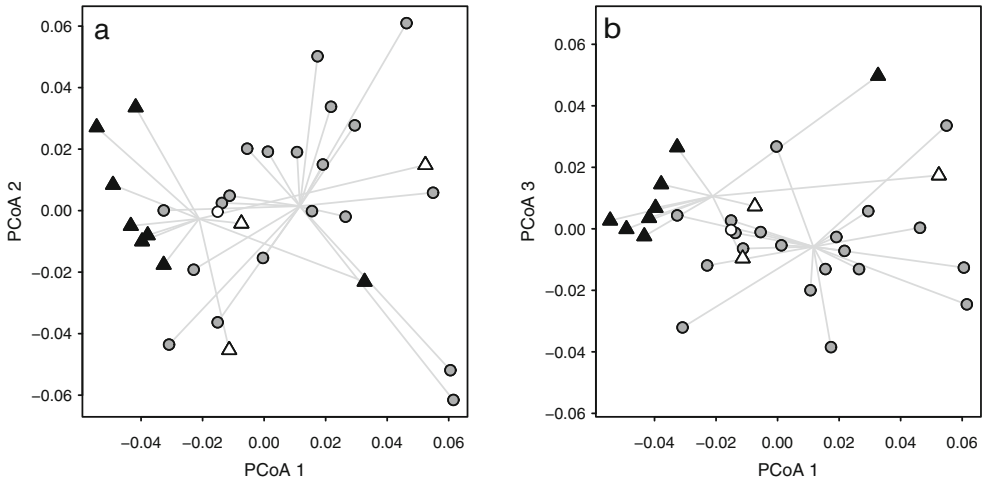

Fig. 3 Principal coordinate ordination plots showing variation between bachelor (circles) and harem (triangles) males. Open symbols indicate individuals that were incorrectly classified via CAP discrimination. Grey lines connect each point to the group centroid. **a** The first axis (PCoA1) explains 25.1% of the total variance and second axis (PCoA2) explains 16.7% of the total variance. **b** The third axis (PCoA3) explains 9.7% of the variation

body size and condition, we did detect enough inter-individual variation to facilitate individual discrimination or recognition, even after accounting for variation due to mating status. The discriminant analysis for individual identity only had a 62% success rate; however, this method is limited in its ability to account for multiple effects simultaneously. Additionally, the compounds included in our analyses represent only a fraction of the total variation present in the chemical composition of the glandular secretion. By using MTBE during the extraction, we specifically targeted non-polar and weakly polar compounds, which include volatile compounds. However, less volatile, polar compounds can also play a key role in communication by modifying the rate at which volatiles are released (Greene et al. 2016; Hurst et al. 1998), influencing the microbial breakdown of signal precursors (Ezenwa and Williams 2014), and facilitating the transport of volatiles to olfactory receptors (Briand et al. 2004; Lazar et al. 2004). For example, house mice secrete highly variable, nonvolatile, major urinary proteins (MUPs) that alter the release of volatile compounds from the urine to create individual odor signatures (Hurst et al. 2001; Roberts et al. 2018).

Testosterone can influence glandular signals (Ebling 1977; Lewis 2009), and testis size is often correlated with circulating testosterone levels in mammals (e.g. Lewis 2009; Morrow et al. 2016; Preston et al. 2012), including bats (Martin and Bernard 2000). However, we did not detect an effect of either the presence of scrotal testes or testes size on the chemical profile of the chest gland. All of our samples were taken during the breeding season, when testes reach their maximal size. In *P. hastatus*, testes regress into the abdominal cavity during

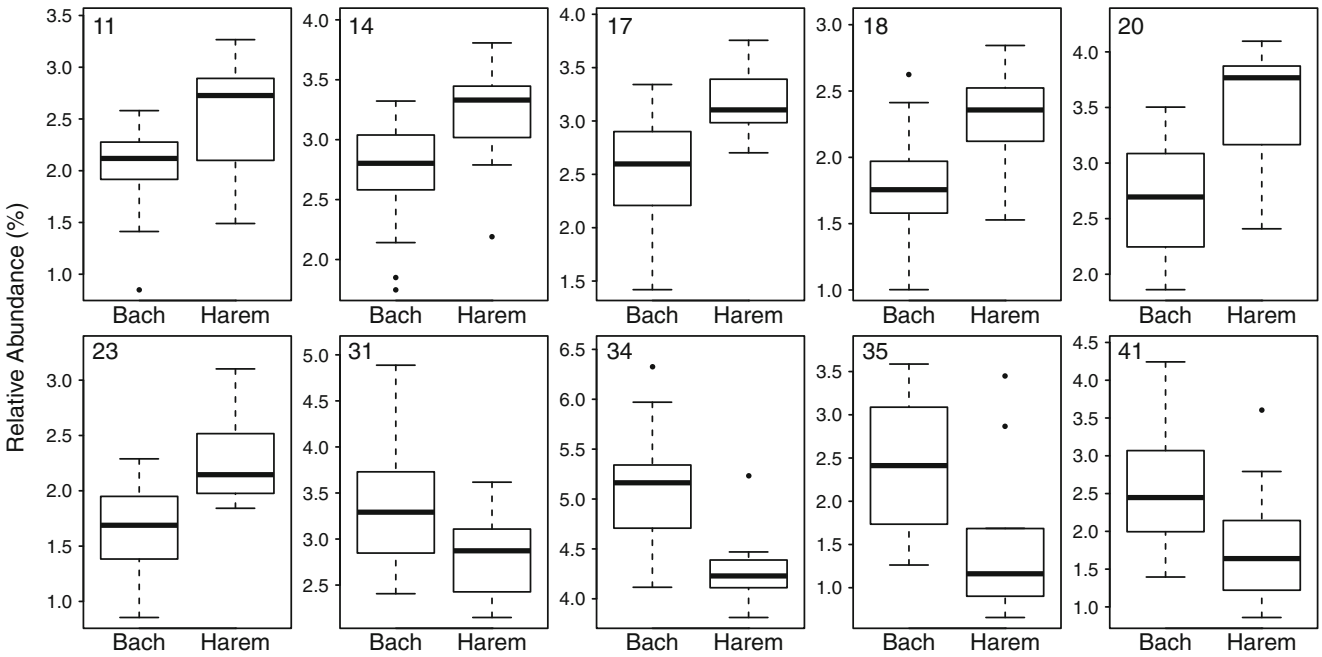

Fig. 4 Relative abundance of the ten compounds significantly associated with a mating status. Numbers correspond to the peak numbers listed in Table 1

Table 3 Effect of individual identity on chemical profiles

Effect	df	SS	pseudo-F	p
	13, 14	0.059	2.949	<0.001
Individual (Status)	10	0.041	2.824	<0.001
Replicate	1	0.001	0.864	0.516
Batch	2	0.007	2.297	0.025

the non-breeding season (McCracken and Bradbury 1981), but we have observed that the chest gland remains active. Tooth wear of the few males with inactive glands during the breeding season, indicates they are young males whose gland may not yet be fully developed. These males were likely born the preceding spring and recently dispersed to join a bachelor group. We did not observe any inactive chest glands during the non-breeding season; at this time pups are clearly distinguished from adults, and all adults are at least 1 year of age. Sampling the gland and hormone levels during different seasons could reveal any seasonal variation in gland composition due to differences in circulating testosterone or reproductive state.

In Trinidad, greater spear-nosed bats mate only during a 3 month period, but harem males actively defend their females throughout the year (McCracken and Bradbury 1981). Because the chest gland remains active year-round and has the ability to signal status, we believe this sexually dimorphic gland is likely to play a role in mate defense. The use of scent in territoriality is well-documented in several mammals (reviewed by Gosling and Roberts 2001), including another harem-forming bat, the greater sac-winged bat (*Saccopteryx bilineata*). *S. bilineata* harem males rub facial gland secretions on the periphery of their harem site in the evening. Because females have already departed by that time, this behavior has been interpreted to be a signal to potential intruders rather than potential mates (Caspers and Voigt 2009). *P. hastatus* harem males mark their roost sites, as evidenced by stains on the cave ceiling at roost sites, as well as the females within their harem (McCracken and Bradbury 1981). When the harem male is present at the harem site, scent marks may be redundant to other visual or vocal cues; however, nightly foraging trips leave females unattended for periods of time. Because olfactory cues will persist in the male's absence, fresh scent marks on females or the roost site could signal his residency to potential intruders. In addition to the presence or freshness of the scent deposit, territorial scent marks often reveal attributes of the territory holder. However, of the male traits we measured, only mating status was significantly associated with variation in the chemical profile.

In many species with stable territories, it is common for individuals to be less aggressive to their neighbors, a phenomenon often referred to as the "dear enemy phenomenon" (reviewed by Temeles 1994). One explanation is that neighbors pose less of a threat than vagrants or floaters because established neighbors already have a territory and are less motivated to encroach upon their neighbors' resources (Jaeger 1981; Temeles 1990). Alternatively, reduced aggression may result from recognition of neighbors and remembrance of previous interactions (Getty 1989; Ydenberg et al. 1988). Although the behavioral outcome may be the same, these different causes rely on different signals. The former requires a signal to assess threat level or categorically discriminate territory holders from floaters. For example, the anal gland secretion of territorial Eurasian beavers (*Castor fiber*) signals a male's mating status, and males spend more time investigating the scent of an unfamiliar subordinate than a more dominant, but equally unfamiliar, male (Tinnesand et al. 2013). In *P. hastatus*, harem males may be less of a threat because they already have females with which to mate, whereas bachelors may attempt to steal copulations or usurp the harem male, and thus may present a greater threat. Therefore, signaling mating status may help harem males avoid confrontation when moving throughout the colony. If this is the case, then we would expect the scent of a bachelor male to elicit a stronger defensive response from a harem male than that of another harem male.

If the 'dear enemy effect' arises from recognition of previous rivals, there must be a mechanism to discriminate familiar and unfamiliar individuals, such as an individually-distinct scent (Carazo et al. 2008; Lopez and Martin 2002; Palphramand and White 2007; Rosell and Bjorkoyli 2002). Within the roost, harem males are likely to experience repeated interactions with their neighbors due to the long-term use of specific roosting sites. We have found that *P. hastatus* glandular secretions have the potential to encode identity, but behavioral testing is needed to determine if this variation is relevant to the receivers. Male pale spear-nosed bats, *Phyllostomus discolor*, which possess the same sexually dimorphic chest glands as *P. hastatus*, are able to discriminate familiar and unfamiliar males from scent marks applied at the roost site (Holler and Schmidt 1993).

In addition to functioning for intra-sexual communication, the chest gland may also produce an inter-sexual signal. In harem-based polygyny, it is often assumed that male-male competition selects for the most competitive mates and any role of female choice is often overlooked. However, growing research on sexual conflict reveals that males preferred by females are not always the most competitive in male-male interactions (Hunt et al. 2009; Okada et al. 2014; Swedell et al. 2014; Wong and Candolin 2005). In *P. hastatus*, opportunities for female choice may arise via copulations with males other than the harem resident given that an estimated 10–40% of pups are fathered by a male other than the harem male (McCracken and Bradbury 1981). Although we found the male chest gland secretion does not reflect male age, size, or condition, it may still provide signals for mate assessment if

individual variation in male scent reveals attributes of male quality that are not correlated with competitive ability, such as genetic compatibility.

Additionally, male chemical signals may directly influence females' reproductive state. While there is no evidence of induced ovulation, sperm storage, or extended reproductive delay in *P. hastatus* (James 1977), parturition is highly synchronized (Porter and Wilkinson 2001). The mechanism underlying this synchrony is unknown, but chemical signals are a likely candidate, given their role in reproductive synchrony in other mammals (deCatanzaro 2015; deCatanzaro et al. 2014; Dodge et al. 2002).

The sexually dimorphic development and activity of the chest gland suggests it has been shaped by sexual selection, but once established, the signal may be co-opted for other social functions. Because all females in a harem are marked by the same male, a group signature scent is inadvertently created. This scent may serve as a redundant signal of group identity, as *P. hastatus* females vocally signal group identity via screech calls while flying to and from feeding sites (Boughman 1997; Boughman and Wilkinson 1998). While the message of the two signals may be redundant, the different modalities have advantages in different contexts. Scent marks are produced only by the males and are long-lasting, and short-range signals can be used in a crowded cave. Screech calls, which are typically given outside the cave to coordinate foraging behavior (Wilkinson and Boughman 1998), are independent of the male, of short duration, and signal over a much longer range. A group scent might facilitate cooperation among members of a long-term group via non-vocal recognition within the roost. Similarly, the greater bulldog bat, *Noctilio leporinus*, forms stable female groups, and females create a group-specific scent by rubbing their heads on the subaxial glands of their group-mates (Brooke 1997). *Noctilio leporinus* females also appear to coordinate movements to and from foraging areas (Brooke 1997). Additionally, adult *P. hastatus* females can discriminate pups from their harem from those of other harems via the pups' isolation calls (Bohn et al. 2007). This group signature is especially important for facilitating cooperative defense of pups (Bohn et al. 2009). Given that pup defense is a costly behavior, especially since groupmates are non-kin, accurate discrimination of group versus non-group is paramount, and signal redundancy may be favored.

Here we have provided chemical evidence to show that male greater spear-nosed bats can advertise both mating status and individual identity by the application of secretions from a scent gland. The observed sexual dimorphism in the gland and scent marking behaviors suggest that this signal serves a role in mate defense, and possibly the acquisition of a harem or the attraction of additional mates. However, further examination of the chemical composition of the secretion and behavioral studies are needed to confirm these possibilities. Exploration of the proximate causes of scent variation, such as hormones, genotype, and microbial community, may also be fruitful.

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Compliance with Ethical Standards

All samples were collected and exported under permits from the Wildlife Section of Forestry Division of Trinidad and Tobago. All methods of capture, handling, and sample collection follow the guidelines set forth by the American Society of Mammalogists and were approved by the University of Maryland Institutional Animal Care and Use Committee (FR-13-77).

Conflict of Interest The authors declare that they have no conflict of interest.

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