



## The role of male forearm crust odour in fringe-lipped bats (*Trachops cirrhosus*)

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### Abstract

Chemical signals can play important roles in communication, and this is especially true for social mammals such as bats. Male fringe-lipped bats (*Trachops cirrhosus*) produce an odorous substance on their forearm, called forearm crust. Only adult males with descended testes produce forearm crust. This is thus a sexually dimorphic odour, which suggests that it is a sexually selected trait. Since males lack a specific gland on their forearm we sought to identify the source of the forearm crust. Our second aim was to test female and male preference for this trait. Based on gas chromatography and mass spectrometry analyses we tentatively identified several compounds that were exclusive to the forearm crust. We found that the chemical composition of the forearm crust was not mainly composed of chest gland secretions or urine. We conducted a y-maze odour preference experiment to test whether adult females and reproductive males preferred the odour of a male with forearm crust compared to the odour of a male without forearm crust. Contrary to our prediction we found that females did not approach the scent of a male with forearm crust more frequently than the scent of male without forearm crust. We found that males with forearm crust, however, preferred the odour of males without forearm crust. Overall our results suggest that in this Neotropical bat species, reproductive males could use odorous signals in the forearm crust to mediate interactions between reproductive males and potentially avoid costly competition for mates or aggression. In sum, our results shed light on the role that chemical mediated signals can play in mammalian social behaviour.

### Keywords

bats, chemical communication, odours, *Trachops cirrhosus*.

## 1. Introduction

Odours often modulate social behaviours. Chemical signals can provide information about an individual's identity, sex, fertility, and dominance status (Mardon et al., 2010; Maruska & Fernald, 2012; Smith et al., 2015). Furthermore, chemical cues can convey information about an individual's overall condition (Johansson & Jones, 2007). For example, chemical signals can communicate information regarding an individual's parasitic infections (Kavaliers & Colwell, 1995; Zala et al., 2004; Mitchell et al., 2017) and dietary condition (Ferkin et al., 1997; Giaquinto et al., 2010). Mammals can use chemicals to mark and defend territories or resources (Gosling & Roberts, 2001; Miller et al., 2003), and to announce their competitive ability (Stockley et al., 2013). Through odorous cues, mammals can discriminate between familiar and unfamiliar conspecifics (Kent & Tang-Martínez, 2014), allowing individual and kin recognition (Mateo, 2003; Thom & Hurst, 2004; Brennan & Kendrick, 2006). Odours can act as chemical fingerprints revealing information about genetic quality (Stoffel et al., 2015) and can also inform about the genetic relatedness between individuals (Charpentier et al., 2008, 2010).

The nocturnal and social lifestyle of bats suggests that they likely rely on olfactory cues to communicate with conspecifics (Dechmann & Safi, 2005). Indeed, in bats, odours have been shown to play a role in species and roost mate recognition (Caspers et al., 2009; Englert & Greene, 2009), offer information of individual identity and colony membership (Safi & Kerth, 2003), and allow lactating females to discriminate offspring (Gustin & McCracken, 1987). Furthermore, the presence of specialized glands (Quay, 1970; Schmidt, 1985; Scully et al., 2000) and osmetrichia (hairs specialized for scent dispersal) are consistent with the importance of chemical compounds for bat communication (Hickey & Fenton, 1987). In many cases, these are sexually dimorphic with only males possessing specialized glands and hairs, suggesting a role in reproduction (Quay, 1970; Hickey & Fenton, 1987). Moreover, that many of these structures are seasonal indicates a potential role in mate attraction, male-male competition, or both (Bloss, 1999).

Males of several species of bats secrete odours from specialized glands (e.g., *Pteropus giganteus*, *P. pumilus*, *P. hypomelanus*, *P. vampyrus*: Wood et al., 2005; *Erophylla sezekorni*: Murray & Fleming, 2008), while other species of bats create odorous cocktails through complex behaviours. For example, greater sac-winged bats (*Saccopteryx bilineata*) perform a 'perfume

blending' behaviour in which reproductive males fill their wing sacs with various bodily fluids and gland secretions (Voigt & von Helversen, 1999). This odorous cocktail plays an important role during courtship displays and mate choice (Voigt & von Helversen, 1999). Within *S. bilineata*, there are seasonal differences in the relative amount of chemical compounds, suggesting that females could assess the reproductive status of males via odours (Caspers et al., 2008). Additionally, the chemical profiles of reproductive male *S. bilineata* vary between individuals in the relative amount of chemical compounds, implying a potential role for individual identification via wing sac odours (Caspers et al., 2008). Male long-nosed bats (*Leptonycteris curasoae*) do not have specialized wing sacs but similarly create an odorous bouquet through stereotypical behaviours by combining bodily secretions and smearing them to create a 'dorsal patch' (Muñoz-Romo & Kunz, 2009). Males with a dorsal patch have descended testes and lower ectoparasite loads than males without a dorsal patch, indicating that this odour could communicate an individual's condition (Muñoz-Romo & Kunz, 2009).

Although several studies have investigated the preference of bats for conspecific odours (Gustin & McCracken, 1987; De Fanis & Jones, 1995; Bouchard, 2001; Englert & Greene, 2009), there are only a limited number of experiments testing the response of individuals towards male specific odours (Caspers et al., 2009; Muñoz-Romo et al., 2011). For example, female *S. bilineata* prefer the odours of male *S. bilineata* to the male odours of the sister species, lesser sac-winged bats (*S. leptura*), suggesting that these odours play an important role in species recognition and in pre-mating isolation (Caspers et al., 2009). Additionally, female *L. curasoae* prefer the odour of a male with a dorsal patch to the odour of a male without a dorsal patch indicating that the dorsal patch is likely a male reproductive trait (Muñoz-Romo et al., 2011). The lack of studies investigating the role of male odours in bats is surprising given the diversity of male odorous signals. Furthermore, the studies that have been conducted have only tested the response of females to male odours. To date, studies have not investigated the response of males to sexually dimorphic male odours.

Male fringe-lipped bats (*Trachops cirrhosus*), the focus of our study, produce an odorous substance on their forearm termed 'forearm crust' (Flores & Page, 2017). This recently discovered odorous ornament is unique to this species as it is unknown in other bat species. Male *T. cirrhosus* produce this substance through a series of stereotypical behaviours: while grooming,

males scratch their body with one hind claw, including around a prominent mid-ventral chest gland, insert the same hind claw into the mouth, and then repeatedly lick the forearm (Flores & Page, 2017). The forearm crust is produced throughout the year, but there is an increase in the numbers of males captured with it from September to December, which coincides with the putative mating period of *T. cirrhosus* (Flores & Page, 2017). The forearm crust is only present in adult males and as a further indication that it could be involved in reproduction all males with this substance had descended testes and enlarged chest glands. Furthermore, the majority of males without forearm crust do not have descended testes (Flores & Page, 2017). Additionally, males with forearm crust had significantly higher body condition indices (body mass (g) / forearm length (mm)) than males without, suggesting that forearm crust could communicate an individual's size and condition (Flores & Page, 2017).

Despite extensive roost observations, there was no evidence of males with forearm crust displaying to females or interacting aggressively toward other males (Flores & Page, 2017). Thus, observations did not shed light on the possible function of the forearm crust in social interactions in *T. cirrhosus*. The aim of the present study was to combine chemical analyses and behavioural experiments to elucidate potential roles of this novel forearm crust. Based on previous observations of males' stereotyped crust application behaviour (Flores & Page, 2017), we predicted that males use secretions from a prominent mid-ventral chest gland to produce the forearm crust. We then compared the compounds found in our samples with those thought to elicit behaviours in other animals to provide insight into the potential function of the forearm crust (Soso & Koziel, 2017).

Our second aim was to investigate whether adult females and reproductive males with forearm crust prefer the odour of a male with forearm crust to the odour of a male without this ornament. Although there is evidence for sexually dimorphic chemosignals in bats (Wood et al., 2005; Murray & Fleming, 2008), bioassays to test the function of olfactory signals have rarely been conducted (cf. Muñoz-Romo et al., 2011). We predicted that females would prefer the odour of a male with forearm crust relative to the odour of a male without it. With respect to males, the response of reproductive males towards odorous sexually dimorphic signals has never been tested in bats, and we considered two alternate outcomes. One possibility was that the forearm crust odour could act as a chemical signal towards competitors (Gosling &

Roberts, 2001), and, hence, would deter other reproductive males. Alternatively, males would not show a preference because the forearm crust is an odorous cue towards receptive females.

Finally, we tested whether the difference in body condition between the test male and the odour donor was correlated with the amount of time the test male spent in the no crust preference area. We predicted that if reproductive males were avoiding each other based on size, then test males would spend more time in the preference area of a male without forearm crust if the difference between body condition between the test male and the odour donor with forearm crust was larger.

## 2. Materials and methods

### 2.1. Study species

Fringe-lipped bats (Phyllostomidae) roost in hollow trees, culverts, buildings and caves (Kalko et al., 1999). They are omnivorous, eating frogs, insects and lizards (Cramer et al., 2001). The mating system of most (92%) leaf-nosed bats (Phyllostomidae) is not known (McCracken & Wilkinson, 2000) and this applies to *T. cirrhosus*. While it has been determined that female fringe-lipped bats give birth to one offspring at a time, the gestation length is unknown (Cramer et al., 2001).

### 2.2. Field methods

We conducted fieldwork in Soberanía National Park in Panamá (Colón province, 9.0743° N, 79.6598° W) from December 2015 to February 2017. This tropical lowland forest is marked by seasonal rainfall (average 2600 mm annually), with a dry season from mid-December to mid-April (Leigh & Wright, 1990).

We captured bats using mist nets (Avinet, Dryden, NY, USA) set over streams, over trails, and at the exits of known roosts. We only included adults in our study, which we identified by the absence of epiphyseal gaps in the phalanges (Brunet-Rossinni & Wilkinson, 2009). We classified females as pregnant by the presence of enlarged nipples and by gentle palpitation of the abdomen (Racey, 2009). We determined if males were reproductively active by the scrotal position and enlarged size of the testes (Racey, 2009). To calculate the body condition index of males, we measured the length of forearm

to the nearest 0.1 mm using a dial calliper (Swiss Precision Instruments, Garden Grove, CA, USA), and we recorded the body mass using a 100-g scale (Pesola, Schindellegi, Switzerland). In this study we calculated the body condition index because this is commonly used to evaluate the condition of bats and has been validated as a predictor for the amount of lipid present (Pearce et al., 2008; Reynolds & Korine, 2009). In addition, this measure is consistent with the measure we used in our previous investigation, allowing direct comparison between the two studies (Flores & Page, 2017). McGuire et al. (2018) recently demonstrated, however, that body mass is a more effective predictor for bats than other common indices (such as body condition index). Given this recent finding we also used body mass as a proxy for condition.

We marked each bat with a passive integrated transponder (Biomark, Boise, ID, USA) to allow for identification of recaptured bats. We released all bats at the site of capture after the preference test or scent collection.

All sampling protocols followed guidelines approved by the American Society of Mammalogists for capture, handling, and care of mammals (Sikes et al., 2016) and were approved by the Smithsonian Tropical Research Institute (STRI) Institutional Animal Care and Use Committee (IACUC No. 2014-1001-2017-2-A4) and the University of Chicago (IACUC No. 72356). All research was licensed and approved by the government of Panama (SC/A-45-16; SE/A 69-15; SE/AH-2-16).

### 2.3. *Chemical analyses*

Prior to collection of forearm crust and chest gland samples, we sterilized cotton swabs and 2 ml glass vials (No. 5182-0715, Agilent, Santa Clara, CA, USA) in dichloromethane (99.99%, Fisher, Fair Lawn, NJ, USA) and dried them at ambient temperature under a fume hood. We collected samples from the forearms of adult male bats for chemical analyses in November and December 2016. We collected samples by swabbing forearms of males with sterilized cotton swabs 10 times. For each male, we also sampled the dorsal body area surrounding the forearm of the bat to control for compounds that could be present in the overall body. Additionally, we collected samples from the enlarged chest gland of males with forearm crust in December 2016 and a urine sample from a male with forearm crust in November 2016. As swabs were sterilized in dichloromethane, a known carcinogen, we did not collect saliva samples (Serota et al., 1986). Sterile gloves were worn to prevent contamination. After collection, we transferred samples into vials that were

capped with silicone septa (No. 5182-0717, Agilent) and added 100  $\mu\text{l}$  of dichloromethane (99.99%, Fisher) as a preservative. All samples were stored at  $-20^{\circ}\text{C}$  until chemical analysis.

We analysed samples with a Gas Chromatography-Mass Spectrometer (Agilent 7890B GC, 5977A MS) equipped with a 30-m column, 250  $\mu\text{m}$  wide. Prior to analyses, we added 1.5 ml dichloromethane (99.99%, Fisher) to soak and cover cotton swabs. We sonicated vials for 10 min at room temperature to remove compounds from cotton. With sterilized forceps, we removed cotton swabs and condensed the extract to a 50  $\mu\text{l}$  concentration by evaporation under a stream of nitrogen. Extracts were transferred to deactivated glass vial inserts (No. 5181-8872, Agilent). We added 104.85 ng of 2-tetradecyl acetate solution (custom made by the Schulz laboratory at the Institute for Organic Chemistry, Technical University Braunschweig, Braunschweig, Germany) as an internal standard.

We injected 2  $\mu\text{l}$  of the sample into the GC and collected data under the following conditions: splitless injection, helium as carrier gas,  $60^{\circ}\text{C}$  inlet temperature, 3-min initial time,  $5^{\circ}\text{C}/\text{min}$  rate,  $280^{\circ}\text{C}$  final temperature, 52-min run time. Blanks of the sampling hardware (vials and cotton swabs) and procedure were run under the same conditions as bat samples. Compounds found in similar quantities in both blanks and samples are not reported. Peaks were matched by the fragmentation patterns of chromatograms using Agilent MassHunter Software (Qualitative Analysis B.07.00, 2015) and by performing mass spectral data base comparisons using the National Institute of Standards and Technology library (2008). We calculated the abundance of the relevant compounds by dividing the peak area of each compounds by the peak area of the internal standard and multiplying this by the concentration of the internal standard.

#### 2.4. Odour preference test

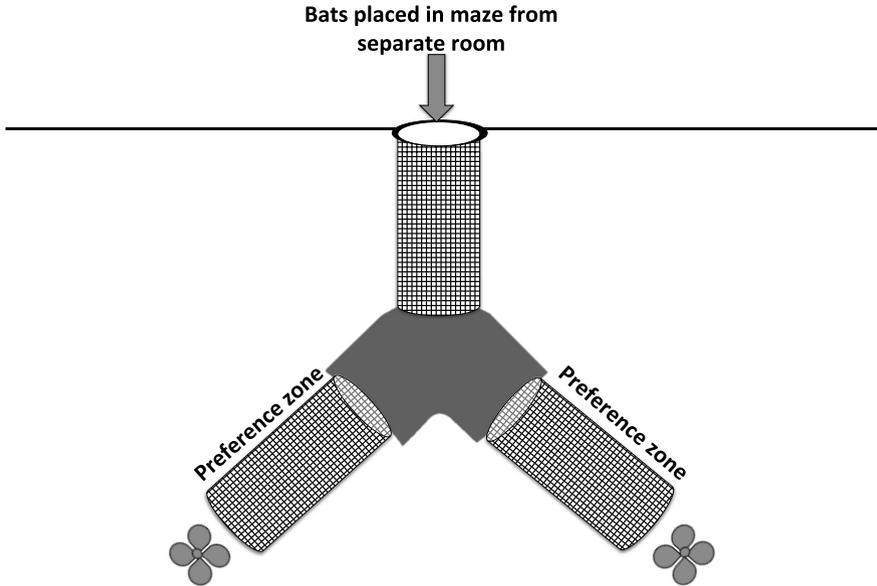
The forearm crust of male *T. cirrhosus* exudes a musky scent to our noses, which permeates the cloth bags used to transport the bats. To collect odour samples we placed individual adult male *T. cirrhosus* in cloth bags for 1 h and used these cloth bags as our odour stimuli (Bonadonna & Nevitt, 2004). We sterilized bags in bleach and stored them individually in ziplock<sup>®</sup> bags prior to odour collection. Bags were made for this experiment and had never previously held other bats. Once we used a bag to collect an odour sample the bag was stored again in a ziplock<sup>®</sup> bag and kept in  $-20^{\circ}\text{C}$  until used in

an experiment. We collected samples from males with forearm crust and descended testes ( $N = 11$ ) and males without forearm crust and without descended testes ( $N = 6$ ) from December 2015 to December 2016. None of the bats included in our preference test were tested with the same set of odour samples. Each odour sample was used once per experiment. Given the small population we did have multiple samples from the same male; however, these were collected during different capture events.

We tested adult females ( $N = 13$ ) from October to December 2016 and adult males with forearm crust ( $N = 12$ ) from October 2016 to February 2017 in a two-choice preference test to examine whether males or females preferred the odour of a male with forearm crust or the odour of a male without forearm crust. We excluded two pregnant and two sub-adult females because we wanted to only include females that were in comparable reproductive stages. All females included in our final analyses were recaptures identified by their transponder number, and hence, we could corroborate they were adults and of reproductive age. Of the 12 reproductive adult males tested, we excluded four males because they failed to investigate either preference zone.

The y-maze consisted of 3 symmetrical arms (L 63 cm; W 12 cm; H 12 cm; angled at  $120^\circ$ ) constructed from plastic garden fence (mesh size 2.54 cm) lined with mosquito gauze enabling the bats to crawl and move (Figure 1). The y-maze arena was placed inside a larger outdoor flight cage ( $5 \times 5 \times 2.5$  m) at the STRI Gamboa field station. Thus, we conducted the experiment at ambient levels of temperature and humidity. The 'starting' arm had an opening to another room where we could place bats into the y-maze to avoid leaving our odour traces in the larger flight cage or y-maze (Figure 1). In each odour-choice arm, a small fan (Model No. FD05004, O2Cool, Chicago, IL, USA) provided a low-noise, controlled airflow. We cleaned the maze (95% ethanol) between trials to remove residue. We chose the side on which each sample was placed arbitrarily. While wearing sterile gloves, we placed samples (cloth bags) at the end of the y-maze arm where bats could come into contact with them.

We conducted the preference test at night between 18:30 h and 1:30 h. Each experiment lasted 15 min. We recorded all movements of the bat during this time using an infrared video camera (Sony DCR-TRV 14E, Sony, Tokyo, Japan) supplemented with infrared lights (IRLamp6, Tucson, AZ, USA). To control for prior familiarity between test subjects and odour individuals, we



**Figure 1.** Odour preference test y-maze diagram. Each y-maze arm was lined with mosquito gauze (hatched pattern). The stimulus odours were arbitrarily placed at the end of each preference zone. A fan directed odours towards the y-maze opening. Bats were introduced to the arena from a separate room.

only used odours from male bats captured  $>2$  km distance from the test subject's capture location. Odour sample location ranged from 2.91 km to 14.62 km from the test subject's capture location. Radio telemetry studies indicate that *T. cirrhosus* have small home ranges, flying an average of 218 m from their day roosts each night to foraging areas averaging 12 ha in size (Jones et al., 2017). To minimize observer bias, all trials were scored by an observer blind to the experimental stimuli.

### 2.5. Statistical analyses

Data were checked for normality using the Shapiro–Wilk test. We tested for differences in the number of chemical compounds between males with and without forearm crust using *t*-tests. To quantify preference, we defined each arm in the y-maze as a preference zone (Figure 1). We analysed the amount of time a bat spent in each preference zone in relation to the total time the bats spent in both preference zones. We compared the proportion of successes ( $>50\%$  of time in a preference zone) with a two-tailed binomial test

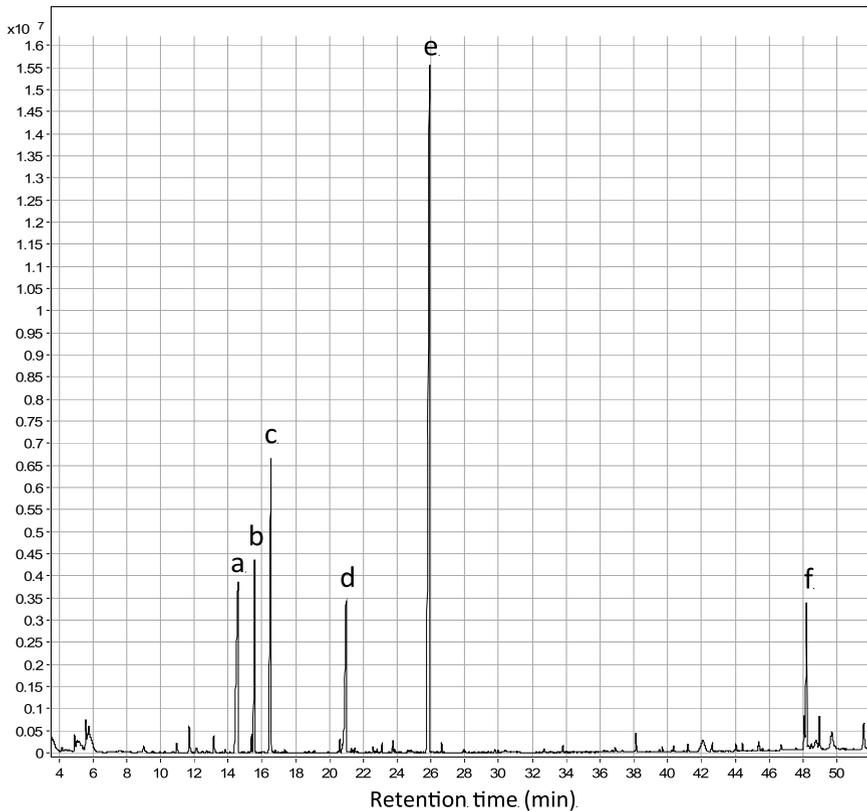
with an expected probability of  $p = 0.5$ . We tested for differences between the duration of time spent on the side of the y-maze with the odour of a male with forearm crust versus the odour of a male without forearm crust with a Mann–Whitney  $U$ -test because data were not normally distributed and transformations were not successful. We used the same test to assess possible differences in the body condition (both body condition index and body mass) of the males chosen by females. We calculated the difference in body condition (both body condition index and body mass) between the test subject male with forearm crust and the odour donor male with forearm crust. We then tested whether there was a relationship between the difference in body condition (both body condition index and body mass) and the duration of time males spent in the no forearm crust preference zone with a Kendall's  $\tau$  correlation. All statistical tests were performed in R (R Core Team, 2013), and the significance level was set  $\alpha = 0.05$ . We report results as the mean  $\pm 1$  SE.

### 3. Results

#### 3.1. Chemical analyses

We collected forearm odour samples from 5 males with forearm crust and 4 males without forearm crust. Of 57 chemical compounds present in forearm samples of males with forearm crust, 25 were tentatively identified. From 29 compounds present in forearm samples without forearm crust, we tentatively identified 13. Males with forearm crust had more substances on their forearm with an average of  $20.6 \pm 3.8$  ( $N = 5$ ) chemical compounds, whereas males without forearm crust had  $13.7 \pm 2.4$  ( $N = 4$ ) compounds, however, this difference was not significant ( $t_7 = 1.42$ ,  $p = 0.20$ ).

We found 58 chemical compounds in control samples (swabs of the area surrounding the forearm) of males with forearm crust and we identified 27 compounds. Similarly, we identified 30 compounds of the 57 found in the control samples of males without forearm crust. Males with forearm crust had an average of  $21.20 \pm 5.98$  ( $N = 5$ ) compounds in the area around the forearm. Whereas, males without forearm crust had an average of  $23.75 \pm 3.33$  ( $N = 4$ ) compounds in the area surrounding the forearm. This difference did not differ significantly ( $t_7 = 0.35$ ,  $p = 0.74$ ). The scent profiles of males with forearm crust (Figure 2, Table 1) mainly included the following substances (abundance): 2-aminoacetophenone ( $\bar{X} = 0.066$  nmol  $\pm 0.017$ ),



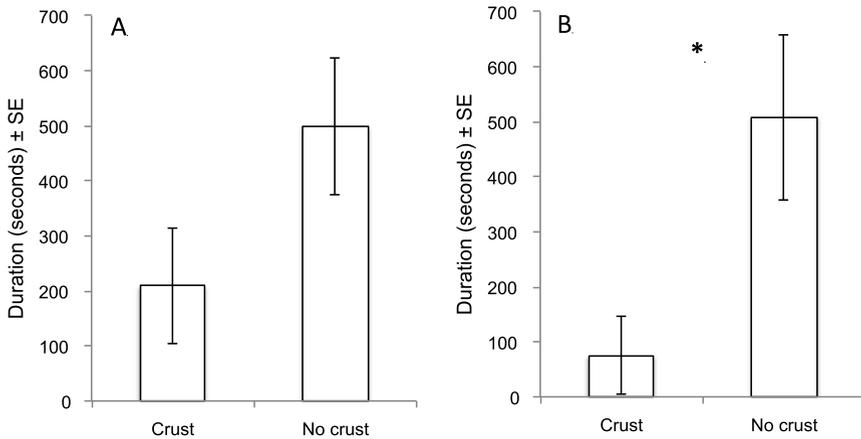
**Figure 2.** Sample chromatogram of forearm crust of an adult male fringe-lipped bat (*Trachops cirrhosus*). a, 5,6-dihydro-6-propyl-2H-pyran-2-one; b, 2-methylquinoline; c, 4-methylquinazoline; d, 2-aminoacetophenone; e, 2-tetradecyl acetate (internal standard); f, cholesterol.

4-methylquinazoline ( $\bar{X} = 0.048 \text{ nmol} \pm 0.029$ ), cholesterol ( $\bar{X} = 0.045 \text{ nmol} \pm 0.009$ ), 5,6-dihydro-6-propyl-2H-pyran-2-one ( $\bar{X} = 0.042 \text{ nmol} \pm 0.03$ ), 2-methylquinoline ( $\bar{X} = 0.032 \text{ nmol} \pm 0.018$ ), and squalene ( $\bar{X} = 0.015 \text{ nmol} \pm 0.00$ ). From the 23 compounds found in chest gland samples of males with forearm crust ( $N = 2$ ) we tentatively identified 6 compounds present in both chest gland samples (Table 1). We found that squalene comprised a large portion of these samples ( $\bar{X} = 2.034 \text{ nmol} \pm 0.112$ ).

The scent profile of the urine sample from a male with forearm crust ( $N = 1$ ) contained erucic acid (among other compounds that we could not identify). As the urine and chest gland samples were collected from males

**Table 1.** Chemical compounds tentatively identified in the forearm crust and in the chest gland secretions of reproductive male fringe-lipped bats (*Trachops cirrhosus*).

Compound	Present in forearm crust (FC)	Present in chest gland (CG)	Molecular weight	Formula	Abundance (nmol)
5,6-dihydro-6-propyl-2H-pyran-2-one	X		140.1	C <sub>8</sub> H <sub>12</sub> O <sub>2</sub>	0.042 ± 0.03
2-methylquinoline	X		143.1	C <sub>10</sub> H <sub>9</sub> N	0.032 ± 0.018
4-methylquinoline	X		144.1	C <sub>9</sub> H <sub>8</sub> N <sub>2</sub>	0.048 ± 0.029
2'-aminoacetophenone	X		163.1	C <sub>8</sub> H <sub>9</sub> NO	0.066 ± 0.017
1,5,9-Undecatriene,2,6,10-trimethyl-,(Z)-		X	192.2	C <sub>14</sub> H <sub>24</sub>	0.133 ± 0.007
6-methyl-5-Hepten-2-yl tigelate		X	210.2	C <sub>13</sub> H <sub>22</sub> O <sub>2</sub>	0.922 ± 0.140
6-methyl-5-Hepten-2-yl angelate		X	210.2	C <sub>13</sub> H <sub>22</sub> O <sub>2</sub>	0.783 ± 0.200
n-Hexadecanoic acid	X	X	256.2	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	0.039 ± 0.004
Cholesterol		X	386.4	C <sub>27</sub> H <sub>46</sub> O	0.045 ± 0.009 (FC) 0.046 ± 0.004 (CG)
Squalene	X	X	410.4	C <sub>30</sub> H <sub>50</sub>	0.015 ± 0.00 (FC) 2.034 ± 0.112 (CG)



**Figure 3.** (A) Female duration and (B) male duration in seconds of time spent in preference zone with odour of male fringe-lipped bat (*Trachops cirrhosus*) with forearm crust or without forearm crust. Significant difference in male preference is denoted by an asterisk (\*).

with forearm crust we could not compare samples of males with and without forearm crust.

### 3.2. Odour preference test

Of the nine females included in our analyses, three (33.3%) chose the scent of a male with forearm crust (binomial test,  $N = 9$ ,  $p = 0.25$ ). Females spent an average of  $706.7 \pm 64.8$  s in both preference zones but spent more time in the no forearm crust preference zone ( $\bar{X} = 497.8 \pm 124.2$  s) than they did in the forearm crust side ( $\bar{X} = 208.9 \pm 104.6$  s, Figure 3a). Females did not differ significantly in the time they spent in the side of the y-maze with the odour of a male with forearm crust versus the odour of a male without forearm crust (Mann–Whitney  $U = 24$ ,  $N = 9$ ,  $p = 0.15$ ). The body condition index of males chosen by females did not differ from the body condition index of the male not chosen (Mann–Whitney  $U = 16$ ,  $N = 9$ ,  $P = 0.07$ ). Neither did the body mass (Mann–Whitney  $U = 1.5$ ,  $N = 9$ ,  $p = 0.06$ ). Three out of nine females chose the crust preference zone first (binomial test,  $N = 9$ ,  $p = 0.25$ ).

Of the eight males analysed, all but one (87.5 %) spent more time near the odour of a male without forearm crust (binomial test,  $N = 8$ ,  $p = 0.035$ ). The one male that preferred the scent of a male with forearm crust showed a distinctive change in behaviour towards the odour sample, crawling back and

forth, and ultimately jumping towards the odour cue to the point of knocking the odour sample and fan. Males spent an average of  $582.6 \pm 132.1$  s in both preference zones, but spent more time in the no forearm crust preference zone ( $\bar{X} = 507.4 \pm 149.6$  s) than they did in the forearm crust preference zone ( $\bar{X} = 75.2 \pm 70.2$  s, Figure 3b). We found that males spent significantly more time in the preference zone with the odour of a male without forearm crust (Mann–Whitney  $U = 5$ ,  $N = 8$ ,  $p = 0.004$ , Figure 3b). We did not find a significant correlation between the body condition index difference between the male with forearm crust making the choice and the male with forearm crust that we used as an odour donor and the duration of time the test subject spent in the no forearm crust preference zone (Kendall's  $\tau = -0.30$ ,  $N = 8$ ,  $p = 0.31$ ). Our findings did not differ when considering body mass (Kendall's  $\tau = -0.38$ ,  $N = 8$ ,  $p = 0.20$ ). Two out of eight males initially chose the crust preference zone (binomial test,  $N = 8$ ,  $p = 0.14$ ).

#### 4. Discussion

We describe the chemical composition and possible function of a newly described odorous ornament in reproductive male fringe-lipped bats. Our results demonstrate that the forearm crust substance is not created solely with secretions from the chest gland or with urine. Additionally, we found that female fringe-lipped bats did not choose the odour of reproductive male *T. cirrhosus* with forearm crust. However, male *T. cirrhosus* with forearm crust significantly preferred the odour of males without forearm crust.

##### 4.1. Chemical composition of forearm crust odours

We identified several compounds in the forearm crust that have been reported in other bat species (Nielsen et al., 2006; Caspers et al., 2009; Muñoz-Romo et al., 2012) and in other mammals (Jorgenson et al., 1978; Wood et al., 2002; Zhang et al., 2005). Cholesterol, and its intermediate squalene, are ubiquitous in mammals (Albone, 1984). Cholesterol is commonly found in mammalian gland secretions (Flood et al., 1989; Burger et al., 2001; Stander et al., 2002; Wood et al., 2005) and was present in our chest gland samples and in all but one of our forearm crust samples. Additionally, we found squalene in several of our samples with particularly high concentrations in the chest gland samples. Squalene has been reported in several flying fox species (*Pteropus hypomelanus* and *P. pumilus*; Wood et al., 2005) as well

as other mammals (Scordato et al., 2007; Zhang et al., 2008) and it has been shown to have multiple roles, from increasing the attractiveness of castrated males to females (Zhang et al., 2008), to acting as a male recognition pheromone (Mason et al., 1989). We suggest that similar to other odorous signals (Scordato et al., 2007; Apps et al., 2012), the molecules with lower molecular weights (4-methylquinazoline, 2-methylquinoline, and 2-aminoacetophenone, Table 1) could be enveloped in a matrix of cholesterol and squalene, which could act as a fixative or controlled-release carrier material (Burger et al., 2001).

Our results provide a tentative chemical characterization that can inform future studies. For example, one of the compounds found in all forearm crust samples, 4-methylquinazoline, has been identified as a component of male sex pheromones in parasitoid wasps (*Nasonia vitripennis*). Although female wasps were not attracted to the odour of this compound alone, 4-methylquinazoline did increase the attractiveness of the odour when combined with other sex pheromones (Ruther et al., 2008). Similarly, components of kissing bug (*Triatoma infestans*) faeces are known to elicit aggregation behaviour (Schofield & Patterson, 1977). Female *T. infestans*, but not males, were attracted to a mixture of 4-methylquinazoline, 2-aminoacetophenone, and other components (Alzogaray et al., 2005). Although most experiments focus on the impact volatile compounds have on insect behaviour, these results can inform future studies in vertebrates, as results suggest that these compounds can elicit a behavioural response.

Contrary to our prediction, we did not find congruence between the compounds in the chest gland and urine samples, and the forearm crust samples. Because males create this forearm crust by licking their forearm, saliva may be an important source of compounds. Compelling evidence for this is found in the unique salivary gland in *T. cirrhosus*. Fringe-lipped bats have accessory submandibular salivary glands that are unlike any other described mammalian salivary gland (Phillips et al., 1987). Although this gland is not sexually dimorphic, the previously identified sexually dimorphic forearm licking behaviour observed only in males could result in a sexually dimorphic odorous substance (Flores & Page, 2017). Saliva is a likely candidate since it is a known chemical signal in mammals and has varying roles, from eliciting receptivity (Melrose et al., 1971), to impacting mate preference (Block et al., 1981; Gray et al., 1984; Talley et al., 2001), to increasing aggression (Taha et al., 2009). Likewise, Nagato et al. (1984) suggest that

salivary gland secretions in male round-eared bats (*Lophostoma silvicolum*) could play a role in species recognition or sexual behaviour. Further studies into the chemical composition of the salivary gland secretions of *T. cirrhosus* are necessary to confirm whether these play a role in the forearm crust production.

#### 4.2. Odour preference test

Contrary to our prediction, female *T. cirrhosus* showed no preference for the scent of males with forearm crust. Two-thirds of females selected the scent of a male without forearm crust. The different responses of female bats to male forearm crust may depend on their sexual receptivity, which we were unable to assess. Voigt & Schwarzenberger (2008) noted that when female *S. bilineata* are not in oestrus they refuse copulations and mating attempts by harem males. Additionally, oestrus in *S. bilineata* is very short, lasting two to four days from late November to late December with the majority of females in oestrus for the first part of December and giving birth in May or June (Voigt & Schwarzenberger, 2008). Similar to *S. bilineata*, female *T. cirrhosus* give birth in May or June (Flores & Page, 2017). Fringe-lipped bats may also have a short oestrous cycle sometime in November and December. Although we tested females in this period, if the window of receptivity is short, it is possible that we might have missed it in some of our test subjects. Future studies should incorporate female receptivity and oestrous cycle either through vaginal swabs or hormone analyses.

Odours can convey a wealth of information on an individual's condition. In this study we used both body condition index and body mass as proxies for an individual's condition (Pearce et al., 2008; Reynolds & Korine, 2009; McGuire et al., 2018). However, we found no significant differences in either measure of body condition between the males selected by females. Alternatively, females might select males based on other qualities. One possibility is that the forearm crust odour could communicate genetic dissimilarity via genes in the major histocompatibility complex (MHC). Some have suggested that odorous signals may provide a more reliable assessment of genotype while evaluating a potential mate than visual or acoustic cues (Johansson & Jones, 2007). Studies in both mammals and birds have demonstrated that females can detect MHC-related odours, preferring individuals that are genetically dissimilar (Spehr et al., 2006; Strandh et al., 2012). These results invite further study into whether *T. cirrhosus* females are choosing males

with a dissimilar genotype via the odours of the forearm crust. Recently, Santos et al. (2016) investigated chemosensory receptor genes (trace amine-associated receptors; TAARs) in *S. bilineata* and demonstrated a correlation between female MHC-dependent mate choice and TAAR diversity. This recent study in bats emphasizes our lack of knowledge about the olfactory system in bats and highlights the need for future work to investigate the sensory and genetic mechanisms underlying mate choice in bats.

We found a significant difference in the choice of males with forearm crust, with seven out of eight males choosing the preference zone with an odour of a male without forearm crust. Furthermore, we had to exclude four males from our analyses because they did not enter either arm, unlike the females that each entered at least one of the preference zones. This perhaps further indicates a male aversion to the forearm crust odours presented. Although an alternative inference is that males with forearm crust find the odours of males without forearm crust to be more interesting, we also observed a distinct change in behaviour in one of the males in our study. While we only observed this escalation of behaviour in one male, we found this behavioural sequence much more likely to signal aggression toward the scent of the male with forearm crust, not simply attraction or interest in the other odour. One possible factor contributing to a male's behavioural response is familiarity. In our study we took this into account by testing individuals from different populations, which had no prior exposure or contact. An interesting direction for future investigation would be to test whether males react more aversely to the scents of unfamiliar versus familiar males as would be expected by the dear-enemy hypothesis (Fisher, 1954; Searcy et al., 2014). Previous work suggests males can assess competitors via odours and avoid larger males (Gosling & McKay, 1990; Gosling et al., 1996). Furthermore, males will avoid the scent of another male if the male being tested is in poor condition (Amo et al., 2012). Hence, our results suggest that male *T. cirrhosus* may evaluate potential opponents through forearm crust odours and may use odours to avoid intrasexual aggression (Luque-Larena et al., 2001; López & Martín, 2011). Although we did not find a significant correlation between the difference in body condition between males and the duration of time spent in the no forearm crust preference zone, future studies could assess other measures of condition (parasite load and immunocompetence) or androgen levels (testosterone). Future studies could also evaluate whether an individual's specific body condition is associated to the specific chemical

composition of the forearm crust (e.g., López et al., 2006). An interesting avenue of research would be to further investigate the relationship between hormone levels and the forearm crust. Testosterone has been correlated with agonistic behaviours and dominance status (Johnston, 1981) and with other behavioural states of increased aggression (Poole et al., 1984). Furthermore, odorous signals can produce graded signals that reflect testosterone levels (Ferkin et al., 1994).

Overall we have tentatively identified the chemical composition of a novel odorous substance in male bats. Previously these compounds were identified as potentially playing a role in mate choice via female choice in other species. However, our results demonstrate the importance of combining chemical analyses and behaviour experiments. Unlike previous results in mate choice studies we suggest that these compounds could instead perhaps play a role in male-male interactions. Future experiments could test the response of *T. cirrhosus* to individual compound concentrations to determine which compounds are eliciting behavioural preferences.

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