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ORIGINAL ARTICLE

The crust of a male: does size matter when females are fertile?

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Abstract

Mammalian odors are frequently sexually dimorphic, with males often exhibiting a stronger, or otherwise distinct, odor relative to females, which can be especially useful for nocturnal species with reduced use of vision. Some male bats exhibit intense odors to attract females and reproduce, presumably as a consequence of a high concentration of testosterone. This usually coincides with the fertile period of females, which can be difficult to recognize visually because female mammals rarely advertise their precise reproductive condition. Recently, a novel odorous crust was discovered on the forearms of adult male fringe-lipped bats, *Trachops cirrhosus*. To understand its reproductive significance, we explored potential relationships between the size of the crust and testosterone concentration, testes size, female reproductive state, ectoparasite load, and body condition. We sampled bats during the mating season and determined crust size, testosterone concentration (plasma), testes volume, precise reproductive status of females (vaginal smears), body condition (ratio of body mass and forearm length), and ectoparasite load. Crust size (0–38 mm^2) was positively correlated both to testosterone concentration (0.7–36.4 ng mL^{-1}) and to relative testes volume (6.3–349.1 mm^3). During the sampling period, vaginal smears confirmed that all females were in estrus. Ectoparasite load in males was significantly lower than in females, although both sexes were in similar body condition. Our results suggest that testosterone concentration is a key factor in the exhibition of a larger crust and presumably a stronger odorous signal to estrus females during the reproductive season.

Significance statement

Male secondary sexual characteristics are critical for attracting females for mating. Male odor, in particular, can be indispensable in nocturnal species, as visual signals may lose their effectiveness under dark conditions. The most important male hormone, testosterone, increases the development of male exclusive traits, including odors. In the Neotropics, reproductive male fringe-lipped bats exhibit large odorous crusts on their forearms. The largest male crusts correspond with the highest concentration of testosterone, a relationship we find in the mating season, when vaginal smears confirm females are fertile. We also show that males with crusts have low ectoparasite loads. Our results suggest that the male crust could potentially reveal critical information. Our research highlights the significance of testosterone in the expression of male exclusive traits.

Keywords Odor · Testosterone · Sexual dimorphism · *Trachops cirrhosus* · Mate attraction · Chiroptera

Introduction

Some of the most conspicuous sexually dimorphic characteristics in mammals are visual such as body size, color, weapons, and ornaments and can reveal health condition and overall quality of individuals (Andersson 1994; Stevens 2013). However, chemical signals (e.g., odors) can also be sexually dimorphic, usually with stronger or distinctive odors in males than in females (Eisenberg and Kleiman 1972; Blaustein 1981; Müller-Schwarze 1983; Andersson 1994; Andersen and Vulpius 1999; Brennan and Kendrick 2006; Stowers and Logan 2010). Chemical signals are especially useful for nocturnal mammals, because visual signals may lose their effectiveness under dark conditions (Altringham and Fenton

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2003; Dechmann and Safi 2005). In addition, diseases might also trigger immunological responses that promptly alter an individual's odor, revealing an individual's overall quality through its chemical profile (Schmidt 1985; Penn and Potts 1998; Bloss 1999; Johansson and Jones 2007).

It has been shown that the development of secondary sexual traits, including the expression of intense male odors, often depends on hormone concentration, specifically testosterone (Ebling 1977; Ferkin et al. 1994; Costa and Paula 2006; Thornhill et al. 2013; Ferkin et al. 2017). Testosterone is also involved in the seasonal activity of male gonads and sperm (Ebling 1977; Andersson 1994; Krutzsch 2000; Martin and Bernard 2000) and can have an effect on specific behaviors, such as courtship, territoriality, and aggressiveness (Hart 1974; Andersson 1994; Greiner et al. 2010, 2011). For example, high testosterone concentrations were found in male capybaras displaying larger scent glands (Costa and Paula 2006), and males with larger scent glands were observed scent marking significantly more than those with smaller scent glands (Herrera 1992). Some studies have shown that the intensity of glandular chemical signals depends on circulating testosterone levels (Ferkin et al. 1994; Thornhill et al. 2013), which in turn can drive differences in the attractiveness of male odors to females (Ferkin et al. 1994; Costa and Paula 2006; Thornhill et al. 2013). For example, Ferkin et al. (1994) observed that controlled higher dosages of testosterone in male meadow voles, *Microtus pennsylvanicus*, increased male attractiveness to females, with the highest testosterone level eliciting the greatest attractiveness.

The exhibition of dimorphic male traits as a consequence of high concentration of testosterone usually coincides with the fertile or estrus period of females, the phase when the female is sexually active (Bronson 1985; Brown 1999). The estrus stage in female mammals is commonly restricted to very short periods of the year, often timed such that parturition coincides with a seasonal increase in food availability, enabling lactation (Bronson 1985; Williams et al. 2017). The precise estrus (i.e., fertile) condition of females can be difficult to quantify because females do not always externally exhibit their reproductive status (Cora et al. 2015; Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017). For many species, to determine precise reproductive status during the estrous cycle of females, it is necessary to examine cell types from the vaginal cavity which reflect hormone concentration (Rasweiler and Badwaik 2000; Cora et al. 2015; Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017).

As in many mammals, sexual attraction and mate selection in bats appear to be mediated by chemical cues produced, stored, and released by skin glands involved in sexual communication (Quay 1970; Mykytowycz and Goodrich 1974), but the mechanisms underlying these chemical signals are poorly understood (Voigt 2013). In addition to the widespread use of chemical signals from specialized glands, many mammals also use non-glandular chemical signals, such as urine, feces, and saliva. A few bat species have been observed to use stereotyped behaviors to blend glandular and non-glandular substances to create complex, odorous signals. Males of the greater sac-winged bat, *Saccopteryx bilineata*, for example, fill small sacs on their wings with bodily fluids (a mixture of secretions from the genital region, gular gland, urine, and saliva, that undergoes bacterial fermentation), and then perform wing displays in front of females to attract them (Voigt and von Helversen 1999; Voigt et al. 2005; Voigt 2013). Male long-nosed bats, *Leptonycteris curasoae*, use feces, saliva, and presumably urine or semen, to create a perfumed dorsal patch on their upper backs which is then used to attract females exclusively during the mating season (Muñoz-Romo and Kunz 2009; Muñoz-Romo et al. 2011a). Recently, a novel odorous crust was discovered on the forearms of male fringe-lipped bats, *Trachops cirrhosus* (Fig. 1), without any evidence of glandular secretion (Flores and Page 2017). This unique dimorphic structure is created only by adult males following a complex series of stereotypical behaviors, in which the male smears both forearms with a thick yellow odorous liquid that appears to come from the mouth. The substance completely dries and becomes a dry yellow odorous forearm crust (Flores and Page 2017), but the relationship between its size and odor intensity remains to be determined. While the odor in and of itself is highly conspicuous to human observers, the crust could also have a visual component. Some bodily fluids (e.g., urine of some rodents) strongly reflect ultraviolet light (Chávez et al. 2003), so it is possible that the forearm crust also serves as a visual signal, perhaps reflecting ultraviolet light. This was also suggested for dorsal patches of male *L. curasoae* (Muñoz-Romo et al. 2011b), but this possibility remains to be studied. While non-glandular odorous features have been found on only a handful of bat species to date (e.g., *Saccopteryx bilineata*

Fig. 1 Male *Trachops cirrhosus* displaying the crust in the dorsal region of the middle section of the forearms; scale bar in millimeters (photo: with permission from Marko König, http://www.koenig-naturfotografie.com), and detail of the crust (photo: this study)

(Voigt and von Helversen 1999), *Leptonycteris curasoae* (Muñoz-Romo and Kunz 2009; Muñoz-Romo et al. 2011b), and *Trachops cirrhosus* (Flores and Page 2017)), we suspect that this type of sexually dimorphic trait may be more common than is currently known, and as the over 1400 bat species worldwide become more intensively studied, we predict more reports of this type of sexual dimorphism.

The simultaneous study of the expression of odorous dimorphic traits, testosterone concentration, and female fertility has not been well addressed in mammals. Bats in particular have received no attention in this respect. The forearm crust of *T. cirrhosus* constitutes a unique opportunity for examining these precise reproductive parameters in both sexes, an approach that is critical to robust understanding of the expression of secondary sexual traits.

Male testosterone concentration is likely a critical component in the expression of the crust, a sexually dimorphic trait that appears as a result of a specific behavior. Since the crust is a key sexually dimorphic character in this species that was long considered to be non-sexually dimorphic (Willig 1983; Willig et al. 1986; Cramer et al. 2001), it is important to understand its relationship with testosterone and other biological parameters, such as testes size (volume), body condition (body mass divided by forearm length), and ectoparasite load. Body condition (an indicator of fat storage, and thus an animal's overall condition) and parasite load are important indicators of an individual animal's fitness and usually related to investment in characters used in sexual display (Green 2001). We hypothesized that crust size in *T. cirrhosus* is related to testosterone concentration because it is a secondary sexual trait produced by a male behavior, more frequently and conspicuously during the mating season (Flores and Page 2017). We thus predict that crust size would show a positive relationship with testosterone concentration and other variables such as testes volume, body condition, and ectoparasite load. We also predict that this positive relationship between testosterone concentration and crust size would coincide with the period of female estrus. Since the display of this dimorphic male trait (the crust) is more conspicuous during the rainy (and mating) season (Flores and Page 2017), our goals were to (1) determine crust size and its relationship with testes size, body condition, and ectoparasite load; (2) assess male testosterone concentration and its relationship with crust size; and (3) confirm the reproductive status of females relative to the mating season in which the crust is conspicuously displayed by males.

Methods

Study species

T. cirrhosus feeds on a wide variety of invertebrate and vertebrate prey including insects, lizards, and frogs, and it roosts in mixed-sex groups (of unknown mating system, McCracken and Wilkinson 2000; Flores et al. 2020) of up to 50 individuals in hollow trees, culverts, buildings, and caves (Cramer et al. 2001). Recent observations from 2012 to 2017, however, indicate smaller groups of individuals (i.e., up to 13) that often switch roosts but have preferred roosting partners (Flores et al. 2020). Most offspring are born at the end of the dry season (Flores and Page 2017), which suggests that individuals reproduce just once per year. Gestation length is unknown, but a five-month period is suspected because the end of the mating season is December and most offspring are born in late May (Flores and Page 2017). Flores and Page (2017) described the stereotypical forearm licking performed by males to create the crust. The male bat scratches its entire body with one of its hind feet/claws, repeatedly puts this same claw into its mouth to lick and nibble it, and then licks one of its forearms several times. No evidence of males courting females was found in observations made in natural roosts or captivity (Flores and Page 2017).

Bat sampling

In our study, bats were captured in their roosts between 10:00 and 13:00 h (except one subadult male captured at night near the roosts) from October to December 2018. Bats were captured in Soberanía National Park, Panama (9.07° N, 79.65° W), a study site with 2175 mm of annual average precipitation and 26.3°C of mean air temperature (Steve Paton, pers. comm.). Several roosts were visited to find *T. cirrhosus*. Females were captured on two occasions. Both times they were found roosting in mixed groups. On October 22, 2018, we captured two females roosting with two males: an adult male displaying a developed crust and a subadult male without a crust. On November 13, 2018, we captured eight females roosting with one subadult male without crust, and what we presume to be an adult male displaying a developed crust that escaped. We think that the bat that we saw escaped during this roost capture attempt was a reproductive adult male due to the intense smell of crust we detected in the roost. Consistent with our speculation, in a six-year study, Flores et al. (2020) found that female *T. cirrhosus* were mostly observed in roosting groups with at least one male with crust. We captured males on six occasions between October 4, 2018 and December 10, 2018, in the following roosting groups: (a) one group of all males, all displaying developed crusts (an unusually intense odor was perceived when approaching this roost), (b) one group of a single male with a crust roosting with two females and a subadult male without crust, (c) a subadult male roosting with females and presumably the male with crust that escaped, and (d) isolated male individuals with crust. Additionally, a subadult male was captured near the roosts at the beginning of the night. Most analyses were performed at the Smithsonian Tropical Research Institute's (STRI) Gamboa field station.

Testosterone determination was conducted at the Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT).

Following capture, bats were held separately in soft cloth bags until processed. Individuals were examined to determine sex, age (adult, subadult, or juvenile), and reproductive status (enlarged nipples or enlarged testes; Racey 2009). Age categories were established based on the relative ossification of the joint bones of the fourth finger of the wing (Brunet-Rossini and Wilkinson 2009). We obtained body mass (Ohaus Cs 200.0 g × 0.1 g), forearm length, and testes measurements (length and width; Fowler Sylvac Ultra-Cal Mark IV/0-150 mm). We used testes measurements to estimate testes volume using the formula of 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; Orr and Zuk 2013; Adams et al. 2018). Bats were marked with passive integrated transponder tags (Biomark, Boise, ID) to allow for identification of recaptured bats in successive capture events. After obtaining all measurements and samples (see below), bats were released at their site of capture.

Forearm crust size

Forearm regions of males were photographed in the moment of capture to quantify crust size directly from pictures by obtaining length and width measurements of the deposited crust. Photos were taken using a digital camera (Canon PowerShot ELPH 340 HS; Canon, Tokyo, Japan) while bats were gently held against a flat surface. A plastic ruler (accuracy 0.1 cm) was consistently placed parallel to the forearm and used for scale (Fig. 1). The area of the crust on the right and left forearms was obtained in square millimeters for each male based on measurements, a method that has been validated previously (Muñoz-Romo and Kunz 2009). An average of these two measures (crust area for the right and left forearms) was obtained for each male and used for analysis as crust size. For males without a crust, we assigned a crust size of zero.

Male testosterone

We collected blood samples (100 μL) into heparinized capillary tubes by puncturing the uropatagial vein with a sterile 26-gauge needle. Samples were immediately centrifuged and plasma was obtained and immediately stored at −80 °C until analysis. Plasma testosterone was determined by an enzyme-linked immunosorbent assay (ELISA) based on the principle of competitive binding (CrystalChem Mouse Testosterone ELISA Kit, Illinois, USA; sensitivity of 0.066 ng/mL; precision CV < 10%). Test procedure was performed according to the manufacturer's instructions.

Female reproductive condition

Precise female reproductive status was determined by the examination of vaginal smears (Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017). These smears were prepared by gently introducing sterile water into the vagina by inserting 1 mm of the tip of a sterile micropipette, following standard procedure outlined by Vela-Vargas et al. (2016). Immediately after obtaining the sample, the obtained suspension was transferred to a microscope slide, dried at ambient temperature, fixed in methanol (99%), and stained using the standard Gram-stain procedure (Vela-Vargas et al. 2016). Smears were examined under a microscope (Olympus BX53). We observed and quantified cellular types to determine specific reproductive condition of the females. Cell type predominance reflects female reproductive condition as follows: parabasal cells (diestrus-anestrus), intermediate cells (proestrus), and superficial cells (estrus) (Cora et al. 2015; Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017).

Body condition and ectoparasite load

Body condition (an indicator of fat storage, and thus an individual's overall condition) and parasite load (number of endo- or ectoparasites) are important indicators of fitness (Reynolds and Korine 2009). These two biological parameters were obtained for both males and females. For each bat, we divided body mass by forearm length, a common metric used in bat studies as a proxy for body condition (Lourenço and Palmeirim 2007; Reynolds and Korine 2009; Flores and Page 2017; McGuire et al. 2018). A recent study by McGuire et al. (2018) asserts that body mass is a more effective predictor of fat storage for bats than other common indices (including the ratio of body mass to forearm length). As such, we also analyzed our data using body mass as an indicator of body condition. Ectoparasite loads were obtained by carefully inspecting all body surfaces of each individual bat and making direct counts of observed streblid flies.

While it was not possible to record all data blind because our study involved the examination of focal animals in the field, to the extent possible, we used blinded methods to minimize observer bias. We measured the crust area, mass, forearm length, and parasite load without knowledge of the male testosterone concentration or female estrus stage. We examined testosterone concentration and vaginal smears using a code to refer to each individual, to ensure these analyses were blind.

Statistical tests

All statistical analyses were performed using R, version 3.5.1 (R Core Team 2018). To assess the relationship between crust size and other biological parameters (testosterone concentration, testes volume, ectoparasite load, body condition, and body mass), we calculated and created plots of the Pearson correlations and the respective 95% confidence intervals through ordinary nonparametric bootstraps of 1000 replicates, contrasting the values of crust size, testosterone concentration, testes volume, body condition, body mass, and ectoparasite load. A Wilcoxon signed-rank test with continuity correction was used to test size differences between the right and left crusts for each male and ectoparasite load between males and females. A Welch two-sample *t*-test was used to test for differences in body condition between males and females.

Data availability

The data generated in the current study are available on github at https://github.com/munozromo/Trachops_STRI.git

Results

A total of 23 individuals of *T. cirrhosus* were captured (13 males and 10 females). From our sample of 13 males captured, three males were subadults and had no crust (i.e., crust size = 0), while ten males (77%) had well-developed (i.e., easily visible) and odorous crusts that varied between 10 and 38 mm^2, located in the dorsal region of the middle section of the forearms (Fig. 1). Considering an average forearm length of 60 mm (SD = 1.5 mm), the largest crust covered approximately one-third of the forearm length, while the smallest covered approximately one-sixth of the forearm length, and three males had no crust at all. Crusts (from the left and right forearms) showed no significant size differences (Wilcoxon signed-rank test with continuity correction, $V = 25.5$, $p = 0.7665$).

Male testosterone, testes size, and crust size

Testosterone concentration was obtained for eight males with crust and a male without crust and varied between 0.7 and 36.4 ng mL^{-1} ($n = 9$, Fig. 2A). For males with crusts, volume of testes varied between 94.5 and 349.1 mm^3 ($n = 10$). Since all males without crust (crust size = 0) had non-enlarged testes ($n = 3$), we assumed a fixed value for their testes volume (6.28 mm^3) for analyses that corresponded to reproductively inactive males from non-mating season to avoid using zero. Pearson correlations were supported by their respective 95% confidence intervals (nonparametric bootstraps of 1000 replicates): crust size showed a strong, positive relationship with both testosterone concentration ($r = 0.68$; 95% CI: 0.0300–0.9262; Fig. 2A) and testes volume ($r = 0.81$; 95% CI: 0.5370–0.9529; Fig. 2B). Testosterone also showed a strong, positive relationship with testes size ($r = 0.78$; 95% CI: 0.0579–0.9524; Fig. 2C).

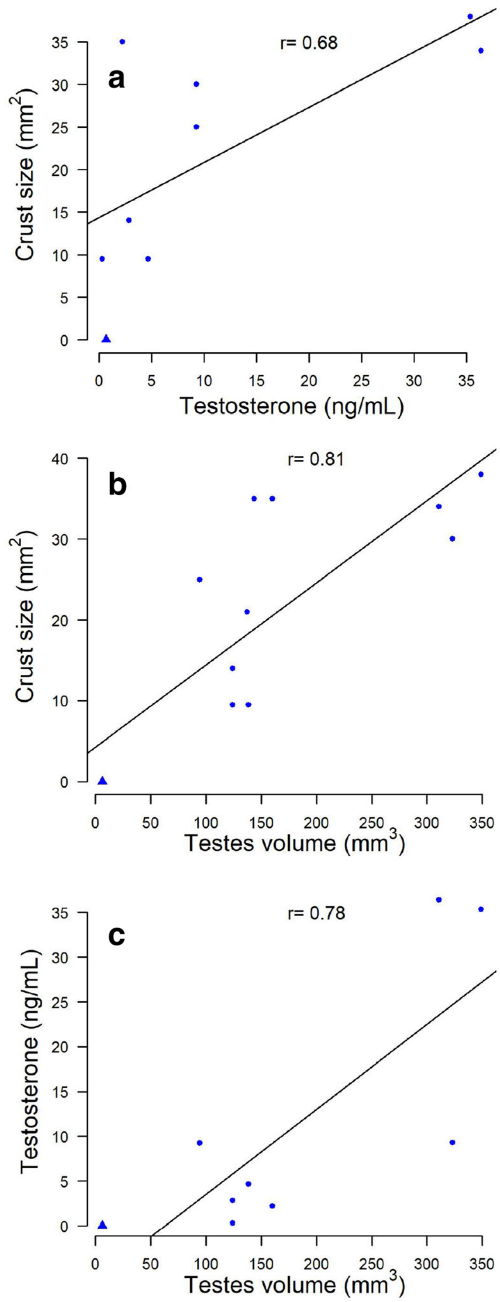

Fig. 2 Relationship between crust size and testosterone concentration (**A**), crust size and testes volume (**B**), and testosterone concentration and testes volume (**C**) in male *Trachops cirrhosus*. Markers (dots and triangles) represent individuals. Forearm crust size in *T. cirrhosus* is positively correlated with both testosterone concentration and testes volume. Testosterone concentration is also positively correlated with testes size. The three males without crust (crust size = 0) and not enlarged testes (6.28 mm^3) are represented with triangles and are overlapped for crust size and/or testes volume. Indicated is *r* (Pearson correlation)

Female reproductive condition

All females were adults ($n = 10$), none were pregnant, and none had enlarged nipples that expressed milk when gently squeezed, indicating that they were not lactating. Vaginal smears of all females examined ($n = 10$) showed 97% superficial cells, 3% intermediate cells, and 0% parabasal cells based on counting the number of cells in the middle of each smear where most cells were observed (total cells observed, $n = 119$). Superficial cells were distinguished as superficial nucleated cells with polyhedral forms (30%), superficial cells with a large cytoplasm and a small pyknotic nucleus (18%), and anuclear superficial (cornified) cells (49%). Predominance of superficial cells and rareness of parabasal/intermediate cells are indicators of estrus state (Carlson and Gese 2008; Cora et al. 2015; Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017). A vaginal smear from a female with no signs of reproductive activity captured during non-mating season (mid-June) was also obtained to compare and validate the procedure, showing predominance of intermediate cells (100% of cells observed) and absence of superficial cells (Fig. 3).

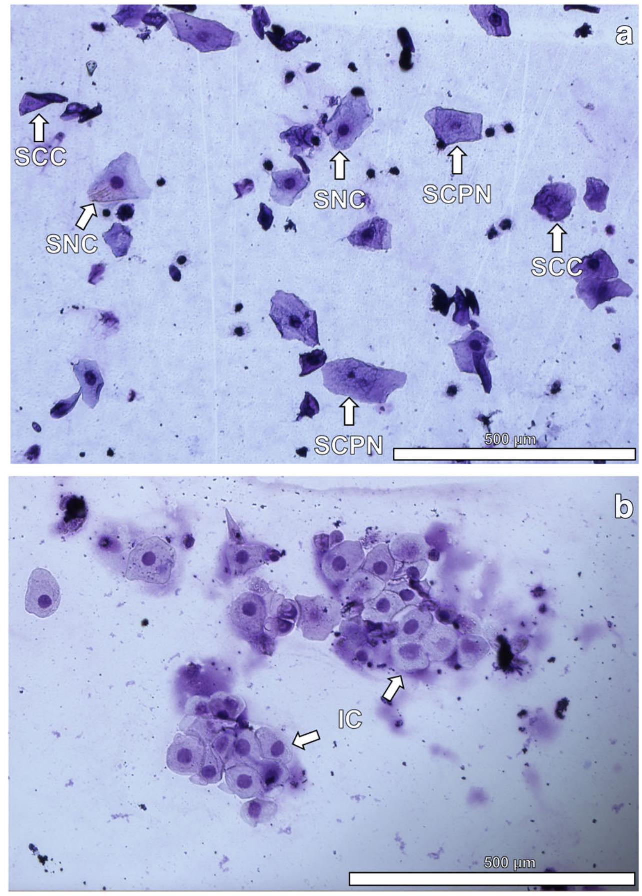

Fig. 3 Superficial nucleated cells (SNC) with polyhedral forms, superficial cells with a small pyknotic nucleus (SCPN), and anuclear, superficial conrnified cells (SCC) observed during the mating season (**A**), and intermediate cells (IC) arranged in groups observed during non-mating season (**B**) in vaginal smears of adult female *Trachops cirrhosus*

Body condition and ectoparasite load

We compared the body condition and ectoparasite load of females and males (males with crust and males without crust pooled). The body condition of females was slightly higher than for males (Fig. 4A), but this difference was not significant ($t = 0.37$, $p = 0.7157$). Males with ($n = 10$) and without ($n = 3$) crust (combined $n = 13$) had significantly lower ectoparasite loads than females ($W = 104.0$, $p = 0.0162$, $n = 10$; Fig. 4B). Although the sample size of males without crust was too low ($n = 3$) for a robust comparison, males with crust ($n = 10$) tended to have higher body condition ($\bar{x}$=0.57) than subadult males without crust ($\bar{x}$ =0.54), and males with crust tended to have lower ectoparasite loads ($\bar{x}$=3) than subadult males without crust ($\bar{x}$ =13). Crust size did not show a strong and significant positive relationship with body condition measured as the ratio of body mass to forearm length ($r = 0.38$; 95% CI: −0.4302–0.9166), or simply as body mass ($r = 0.40$; 95% CI: −0.3191–0.8865), both used as indicators of body condition. Crust size tended to show a non-significant negative relationship with ectoparasite load although it was not highly correlated ($r = -0.43$; 95% CI: −0.9011–0.6193).

Discussion

In this study, we explored the relationship between male crust size and other biological traits, such as male testosterone concentration, testes volume, body condition, and ectoparasite load, and simultaneously confirmed the reproductive status of females. These features are discussed below in the context of the expression of secondary sexual traits.

The crust

The crust of *T. cirrhosus* is an odorous sexually dimorphic trait in adult males that is spread over the dorsal surface of the forearm. When it dries, it is found on the middle dorsal section of the forearm, and it has an intense odor in both the wet and dry states. Of the individuals examined in this study, the largest crust observed occupied approximately a third of the total forearm length, and although the absence of crust was considered as size zero, the smallest crust observed occupied approximately a sixth of the total forearm length. For each individual male, the area of the crust covering the left and right forearms was approximately equivalent, suggesting that males apply equal amounts of substance for each of their two forearms. The specific position of the odorous crust in the body suggests it could be involved in a wing display, or could be the result of smearing a thick substance in the most flat, accessible, and "naked" part of the body.

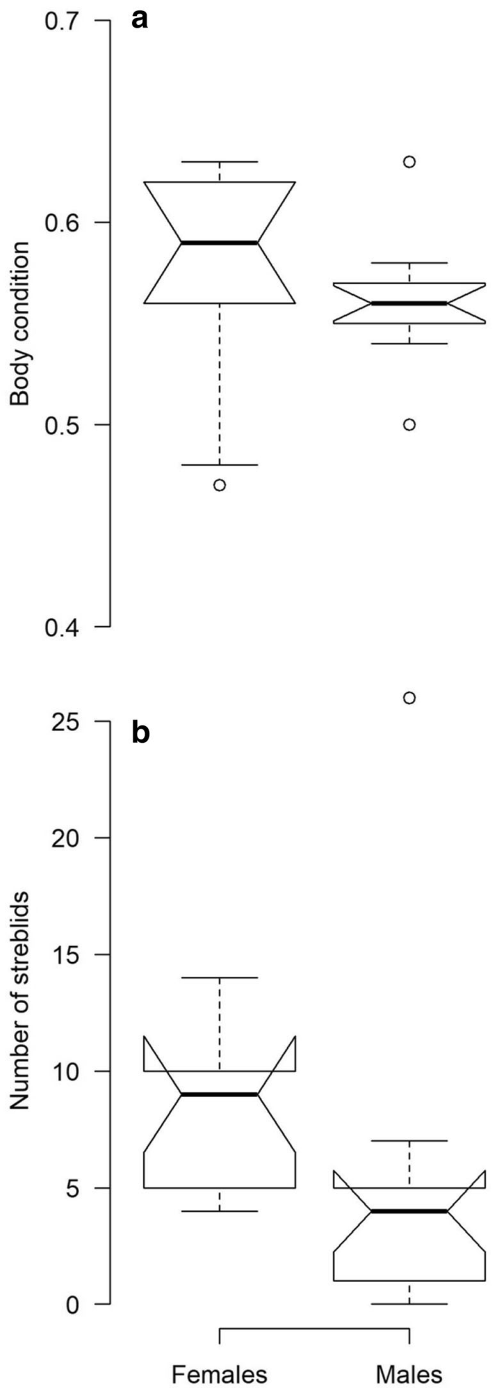

Fig. 4 Body condition (**A**) and ectoparasite load (**B**) of males and females. Females had a non-significantly slightly higher body condition than males (with and without crust), although males had a significantly lower ectoparasite load compared to females. The boxes show the sample median, and the first and third quartiles, and the whiskers show the minimum and the maximum—excluding outliers

The crust, testes size, and testosterone concentration

The range of testosterone concentration found in male *T. cirrhosus* was consistent with values for other species of bats and other vertebrates (Gustafson and Shemesh 1976). Strong variation in testosterone concentration between males is consistent with that reported in sac-winged bats (Greiner et al. 2011). Greiner et al. (2011) found that male sac-winged bats with a female group (harem) had higher levels of testosterone than peripheral males without females, during the weeks of mating season. We found a positive relationship between crust size and testosterone concentration. This pattern was consistent across all individuals except one, which showed an opposite relationship. The increase in the concentration of testosterone could be driving increased crust production and/or increased frequency of smearing behavior, points that require further investigation with increased sample sizes and additional behavioral observations. For example, the two highest testosterone concentration values corresponded to adult males captured in the all-male group, and the third highest value of testosterone concentration corresponded to a male captured with females, although its testosterone concentration was similar to that of the male roosting alone. It is unknown whether these isolated males displaying crusts were also roosting with other individuals (presumably females) that escaped while we were approaching the roosts. Likewise, the largest crusts (≥ 30 mm^2) observed during the study corresponded to three adult males within the all-male group and to the male captured with females. Smaller crusts (< 25 mm^2) were observed in adults of the all-male group and in isolated adult male individuals.

Our observation of adult males with the highest testosterone concentration values and the largest crusts captured roosting together as an all-male group suggests that reproductive male *T. cirrhosus* with forearm crusts could be joining together to perform odorous displays during the mating season. Although this result may explain why we perceived such an unusually intense odor while approaching to this roost, additional observations in future research are critical to fully understand the formation of all-male groups with enlarged testes and developed crusts. Flores et al. (2020) recently reported 18 observations of all-male groups in a period of six years, with at least one reproductive male with a forearm crust in each group.

In our study, the relationship between testosterone and crust size was further supported by a positive correlation between crust size and testes volume. Consistent observations were made by Herrera (1992), who found the size of the scent gland to be positively correlated to testes size in adult male capybaras, *Hydrochoerus hydrochaeris*.

The first and third largest measurements of testes volume (> 300 mm^3) in our study corresponded to two males in the all-male group, and the second to the male captured with females; these three males with the largest testes size also displayed the largest crusts (≥ 30 mm^2). This consistency was expected, as testes produce the primary male sex hormone, testosterone (Krutzsch 2000; Martin and Bernard 2000).

Flores et al. (2019) did not observe female attraction to male odor (i.e., to a cotton bag in which a male displaying crusts had been held for one hour). They did, however, find that, given a choice test, males with forearm crusts were preferentially attracted to the odor of unfamiliar males without forearm crust over the odor of unfamiliar males with a forearm crust. Flores et al. (2019) posit that the odorous crust could serve to mediate aggressive interactions between reproductive males, possibly allowing individuals to avoid costly male-male competition. Given our observation of multiple males forming an all-male group with developed crusts, it would be very interesting to further investigate the role of the crust in male behavior.

The crust, body condition, and ectoparasite load

The assessment of body condition indicated the males with crust tended to have higher condition than males without the crust, which is consistent with results reported by Flores and Page (2017). Although a male in high condition (> 0.6) was captured roosting alone, the highest body conditions were observed in the only adult male captured with females and in one of the males forming the all-males group. Females tended to be in slightly better condition when compared to males in general. This difference may be partially explained by the physiological preparation for pregnancy, which would demand an optimum body condition in females (Wilson 1979; Racey 1982; Heideman 2000). This difference may also reflect the cost of developing and maintaining forearm crusts, a presumably energy-demanding phenomenon that involves the physiological cost of producing the substance (Ferkin et al. 2017) and the behavioral cost of applying it (Flores and Page 2017), a process that requires coordination and consistency to build and maintain balanced crusts.

Females showed higher counts of ectoparasitic streblid bat flies, which is consistent with studies of Neotropical bats (Patterson et al. 2008). Patterson et al. (2008) stated that higher levels of parasitism among female bats could be related to their generally higher survivorship and enhanced probabilities of lateral and vertical transmission of host-specific parasites. Patterson et al. (2008) found sex differences in total numbers of flies in 21 species of Neotropical bats, and in 16 of these, females carried disproportionately heavy loads. It is important to consider, however, that sexual differences between ectoparasite loads may also be affected by roosting behavior (Lewis 1995). It is still unknown whether females of *T. cirrhosus* roost more closely together (i.e., in physical contact) or more consistently in the same roosts. Since at least some ectoparasites of *T. cirrhosus* are puparious (i.e., females deposit pupae on roosting surfaces), individuals that move frequently among roosts are less likely to have bat flies. Thus, differences in roosting behavior between males and females could also influence this result. Although male-female groups are commonly observed in *T. cirrhosus* (Flores et al. 2020), males tend to disperse more frequently (Halczok et al. 2018).

The crust and female reproductive status

The remarkable predominance of superficial cells in vaginal smears of all females examined indicate that the females were in estrus, thus confirming the putative mating season suggested by Flores and Page (2017). The exhibition of the dimorphic forearm odorous crust associated with high concentrations of testosterone coincided with the fertile period of females (Martin and Bernard 2000; Flores and Page 2017). The fertile period of individual females of the greater sac-winged bat, *Saccopteryx bilineata*, was estimated to last only two days (Voigt and Schwarzenberger 2008), but the exact length of the fertile period in females of *T. cirrhosus* remains uncertain, requiring detailed monitoring of individuals over time. It is also possible that fertility periods in female *T. cirrhosus* may not be perfectly synchronized. Future studies could sample and examine vaginal smears repeatedly over time to monitor females to assess other potential aspects of their reproductive behavior, such as post-copulatory selection (Hosken 1997; Wilkinson and McCracken 2003; Orr and Zuk 2013, 2014).

Although it is possible to observe males with crusts throughout the year, there is a marked difference in crust expression during the mating season, the period in which we focused our study. During the mating season, there is an increase in males with forearm crusts, with crusts showing maximum development during this period (Flores and Page 2017). The rest of the year, females are pregnant (January–May) and lactating/nursing (June–September), with behavior related to pregnancy/nursing pups seeming to take precedence over male crust/female estrus. Determining precise reproductive condition through vaginal smear examination has proved to be a straightforward procedure that can produce clear and unambiguous results (Vela-Vargas et al. 2016; Castillo-Navarro et al. 2017). Confirming that females are in estrus during the period in which the crust is most conspicuously developed (Flores and Page 2017) strengthens the evidence supporting our hypothesis that the forearm crust of males is a sexual trait, playing a key role in the reproductive behavior of *T. cirrhosus*.

To our knowledge, our study is the first multi-disciplinary approach combining male androgen concentration, female receptivity, and their relationship to a secondary sexual trait in bats. Our research highlights the significance of testosterone in the expression of a sexually dimorphic trait and its importance in the context of such traits in other mammal and vertebrate groups. Moreover, this study constitutes a novel, focused contribution expanding our understanding of a recently discovered, unusual, complex secondary sexual trait which may be critical for the reproduction of an important Neotropical bat species.

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Compliance with ethical standards

Competing interests We declare we have no competing interests.

Ethics approval The research with bats was carried out in accordance with permits from the STRI Institutional Animal Care and Use Committee (IACUC: 2018-0901-2021) and the Panamanian Ministry of the Environment (Ministerio del Ambiente: SE/A-78-18). Procedures were also consistent with the guidelines of the American Society of Mammalogists for the capture, management and care of mammals (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists 2016), and the Association for the Study of Animal Behaviour.

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