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Scent Marks Signal Species, Sex, and Reproductive Status in Tamarins (*Saguinus* spp., Neotropical Primates)

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Abstract

Olfactory communication is an important mediator of social interactions in mammals, thought to provide information about an individual's identity and current social, reproductive, and health status. In comparison with other taxa such as carnivores and rodents, few studies have examined primate olfactory communication. Tamarins (Callitrichidae) conspicuously deposit odorous secretions, produced by specialized scent glands, in their environment. In this study, we combined behavioral and chemical data on captive cotton-top tamarins, *Saguinus oedipus*, and bearded emperor tamarins, *S. imperator subgrisescens*, to examine the role of olfactory communication in the advertisement of species, sex, and reproductive status. We observed no difference in scent-marking behavior between species; however, females marked more frequently than males, and reproductive individuals more than non-reproductive ones. In addition, tamarins predominantly used their anogenital gland when scent-marking, followed by the suprapubic gland. We collected swabs of naturally deposited tamarin anogenital scent marks, and analyzed these samples using headspace gas chromatography-mass spectrometry. Despite a limited sample size, we established differences in tamarin anogenital mark chemical composition between species, sex and reproductive status, and identified 41 compounds. The compounds identified, many of which have been reported in previous work on mammalian semiochemistry, form targets for future bioassay studies to identify semiochemicals. Our non-invasive method for collecting deposited scent marks makes it a promising method for the study of olfactory communication in scent-marking animal species, applicable to field settings and for the study of elusive animals.

Key words: callitrichids, gas chromatography-mass spectrometry, olfactory communication, primates, semiochemicals, solid-phase microextraction

Introduction

Olfactory communication in mammals involves semiochemicals which can give conspecifics information on an individual's identity, as well as their social, reproductive, and health status (Brown and Macdonald 1985; Wyatt 2014). Semiochemicals produced by the sender are released to the environment, either via passive exudation of body odors or excretions, or via active deposition of scent gland secretions during scent-marking, and can constitute inter- and intra-specific cues and signals (Wyatt 2014). Chemical profiles of

mammalian scent gland odor secretions can convey information on species (e.g. in owl monkeys, *Aotus nancymaae* and *A. azarae*, Spence-Aizenberg et al. 2018; and sympatric Siberian weasels, *Mustela sibirica*, and steppe polecats, *M. eversmanni*, Zhang et al. 2002), group (e.g. in mandrills, *Mandrillus sphinx*, Vaglio et al. 2016; and meerkats, *Suricata suricatta*, Leclaire et al. 2017), sex (e.g. in mandrills, Setchell et al. 2010; and giant pandas, *Ailuropoda melanoleuca*, Hagey and MacDonald 2003; Yuan et al. 2004), reproductive status (e.g. in Coquerel's sifakas, *Propithecus coquereli*, Greene and Drea 2014; and cotton-top tamarins, *Saguinus oedipus*, Washabaugh and Snowdon 1998), and individual identity (e.g. in common marmosets, *Callithrix jacchus*, Smith et al. 2001; and European badgers, *Meles meles*, Buesching et al. 2002a, 2002b). Furthermore, specialized scent glands may also contain different semiochemicals, allowing different cues and signals to be conveyed via one or another of the glands, as suggested in ring-tailed lemurs, *Lemur catta* (Scordato et al. 2007; Greene et al. 2016a). Although primates have historically been considered to have a poor sense of smell (Heymann 2006), researchers are increasingly recognizing the prominent role of olfactory communication in this taxon's sociosexual systems (Snowdon et al. 2006; Drea 2015) and ecology (Kemp and Kaplan 2012; Nevo and Heymann 2015). However, few studies have examined the chemical composition of primate chemosignals, in comparison with other mammalian taxa such as carnivores and rodents (Heymann 2006).

Tamarins (family Callitrichidae) are small Neotropical primates inhabiting a variety of habitats, from tall primary forests to farmlands (Sussman 2003). In the Callitrichidae, semiochemicals are produced via three specialized scent glands, of comparable histology, in the anogenital, suprapubic, and sternal regions of the body (Perkins 1966; Epple et al. 1993; Fontani et al. 2014). Glandular secretions are conspicuously deposited on branches and lianas (scent-marking), or on the body of a conspecific (allomarking; Epple 1974). Previous work has attributed several functions to callitrichid scent-marking, including the advertisement of individual traits such as sex as well as reproductive and dominance status (Smith 2006); territorial advertisement and defense (Lazaro-Perea et al. 1999); and spatial orientation and signaling of food resource location (Miller et al. 2003). Varying observations across callitrichid species and across study conditions (i.e. captive or wild) suggest that scent-marking is likely to occur in a variety of contexts (Snowdon and Ziegler 2020). Innovative methodological approaches, such as the development of modern analytical chemistry techniques for semiochemical analyses, and that of functional brain imaging for observing direct responses to the presentation of scents, are offering new insight into the mechanisms involved in callitrichid chemical signaling.

Our study examined behavioral and chemical aspects of olfactory communication in cotton-top tamarins, *S. oedipus*, and bearded emperor tamarins, *S. imperator subgrisescens*. The objective of the study was to assess the role of scent-marking in the advertisement of species, sex and reproductive status in these two tamarin species. We evaluated differences in 1) their scent-marking behavior and 2) the chemical composition of their anogenital scent marks. Few studies have directly compared scent-marking behavior between different species of callitrichids. Geoffroy's saddleback tamarins, *S. fuscicollis*, and Weddell's saddleback tamarins, *Leontocebus weddelli*, have been observed to mark more frequently than sympatric moustached tamarins, *S. mystax* (Smith 1997; Heymann 2001), and emperor tamarins, *S. imperator* (Watsa, pers. com.). This could originate from differences in the ecology, phylogeny, and social organization between these sympatric species (Heymann 2001). This study provides

a good within-genus comparison of tamarin chemosignaling. The two species studied, cotton-top and emperor tamarins, are cogeners; they show a number of similarities in their ecology and social organization (e.g. diet, group size, reproduction), but also some differences (e.g. emperor tamarins form mixed-species groups with other sympatric tamarins while cotton-top tamarins do not, and emperor tamarins are principally canopy dwellers while cotton-top tamarins are understory dwellers; Rylands 1993; Sussman 2003; Rylands et al. 2016). Here, we predicted that cotton-top and emperor tamarins would show similar rates of scent-marking as they are cogeners and are similar in their ecology. Nevertheless, we predicted that the chemical composition of their scent marks would differ, reflecting a species-specific odor profile important in species recognition, especially for taxa that form mixed-species groups. Dominance and reproductive status are highly entwined in callitrichids, owing to their cooperative breeding system (Huck et al. 2005). Several studies on this taxon have shown that female callitrichids may communicate their reproductive state via odor cues (reviewed in Snowdon et al. 2006; Ziegler 2013), and scent-marking is thought to play a role in the reproductive inhibition of callitrichid subordinate females, occurring through both behavior and chemical cues from the dominant female (reviewed in Beehner and Lu 2013). Therefore, we predicted that reproductive females would scent-mark more often, and their deposited marks would be chemically different from those from subordinate females and males. This would provide a means of indicating their reproductive status to potential mates and inhibiting ovulation in the subordinate females of the group.

Materials and methods

Study sites and species

Scent-marking was studied in captive cotton-top tamarins, *Saguinus oedipus* (Linnaeus 1758; *n* = 10), and bearded emperor tamarins, *S. imperator subgrisescens* (Deville 1849; *n* = 8) housed at Twycross Zoo (TZ), Paradise Wildlife Park (PWP) and Drayton Manor Park (DMP) in the United Kingdom. All tamarin groups were kept in large enclosures composed of 2–3 indoor areas and 1–2 outdoor areas, furnished with diverse substrates such as branches, ropes, platforms and potted plants. Group composition and time of study are given in Table 1. Individuals in each group were classified as reproductive adults (i.e. fully sexually mature individuals), subordinate adults (i.e. offspring of the reproductive pair, >18 months old, probably sexually mature but not having reproduced), and juveniles (i.e. offspring of the reproductive pair, less than a year old). None of the individuals were receiving contraceptives. Two categories of reproductive status were considered: reproductive (i.e. the reproductive pair) and non-reproductive (i.e. subordinate and juvenile offspring), which did not change during our study period. The study sites are members of the British and Irish Association of Zoos and Aquariums (BIAZA). This project was approved by the Faculty of Science and Engineering Departmental Research Ethics Panel at Anglia Ruskin University and received support from BIAZA. It adheres to the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates, and follows the Animal Behavior Society Guidelines and the American Society of Mammalogists' Guidelines on Wild Mammals in Research.

Behavioral data collection

Each tamarin group was observed for 50 h on 10 days distributed over 3–4 weeks, which covered a full female estrous cycle (i.e.

Table 1. Number of recorded scent-marking events and scent mark swabs collected from each individual composing the four tamarin groups included in the study

Species studied	Site and time of study	Group composition	No. scent marks	No. samples
Cotton-top tamarin, *Saguinus oedipus*	Paradise Wildlife Park, Sep. 2017	Reproductive ♂	20	2
		Reproductive ♀	44	3
		Subordinate ♂	8	1
		Subordinate ♀	85	4
		Juvenile ♂	4	1
		Juvenile ♀	0	0
	Drayton Manor Park, Jan. 2018	Reproductive ♂	33	0
		Reproductive ♀	227	4
		Juvenile ♂1	13	0
		Juvenile ♂2	5	0
Emperor tamarin, *Saguinus imperator*	Twycross Zoo, Apr. 2017	Reproductive ♂	21	1
		Reproductive ♀	51	6
		Subordinate ♂	25	2
		Subordinate ♀	28	4
		Juvenile ♂	6	1
		Juvenile ♀	8	1
	Drayton Manor Park, Feb. 2018	Reproductive ♂	27	1
		Reproductive ♀	102	3
Total	4 groups	18 individuals	707	34

23.6 days in *S. oedipus*, French et al. 1983; around 15 days in *S. imperator*, Rylands and Mittermeier 2013). Daily observation time was 5 h, divided into five 1-h bouts at variable intervals between 09:00 AM and 16:40 PM. A single observer (ACP) collected all observational data, thus limiting the variability of the recordings (Martin and Bateson 2007). Prior to data collection, at least 1 day of observation was spent habituating the primates to the observer's continuous presence, and for the observer to learn to differentiate individuals. Individuals were differentiated on the basis of size and natural markings. All instances of scent-marking were recorded for all individuals during each bout of observation. A scent-marking event was defined as a rubbing movement involving the anogenital, suprapubic or sternal scent gland (*sensu* Heymann 2001). Marking duration was variable; consecutive scent marks deposited on the same spot and by the same individual within 2 min were recorded as a single event. For each scent-marking event, day of observation, the identity of the marker (i.e. species, study site, group, sex, reproductive status, and individual ID) and scent gland used (i.e. anogenital, suprapubic, or sternal), were recorded.

Odorant sample collection

A subset of the recorded scent marks was sampled when access inside the animals' enclosure was possibly less than 10 min after deposition. Depending on the zookeepers' availability, a number of samples were additionally collected outside the behavioral observation time. Only scent marks that had not been overmarked or stepped on by other tamarins between time of deposition and time of collection were collected. All but two of the samples collected were anogenital scent mark depositions, as animals in our study marked much more frequently with their anogenital gland than with their suprapubic and sternal glands. Therefore, only anogenital scent gland samples were further analyzed for chemical composition. A total of 34 tamarin samples were included in the chemical analysis (Table 1).

Collection of odorant samples was performed by swabbing the branch spot (usually a wet mark was visible to help locate the secretion) repeatedly 3–6 times, using a clean 1 cm² square of viscose gauze—hereafter referred to as swab—held by clean forceps. Swabs were kept individually in 4 mL glass chromatography vials closed by a screw-top polytetrafluoroethylene septum lid. Prior to use, both vials and swabs were washed in HPLC-grade methanol and pentane (ACROS Organics, London, UK), then baked at 130 °C for 30 min prior to use, as recommended by Birkemeyer et al. (2016). After collecting the secretion, the swab was quickly returned to its vial and closed, and the forceps were wiped on clean gauze with pentane. Sample vials were kept in an insulated cool box filled with frozen gel packs at a temperature close to 0 °C (recorded by an automatized temperature data logger), then transferred to a freezer onsite (−15 °C at DMP and PWP; −20 °C at TZ) within 2 h. At the end of each data collection period, samples were transported in the cool box to Anglia Ruskin University where they were stored at −80 °C until analysis.

Chemical analyses

Scent mark swabs were analyzed one-by-one using headspace solid-phase microextraction–gas chromatography-mass spectrometry (SPME–GC–MS). Each sample was retrieved from the freezer just before analysis, and placed in a heat block at 40 °C for an equilibration period of 10 min. Samples were extracted using a 65 μm polydimethylsiloxane/divinylbenzene StableFlex SPME fiber (Supelco, Bellefonte, PA, USA) for a period of 30 min at 40 °C. The sample-loaded fiber was then manually injected at 250 °C into the injection port of a Clarus 500 GC (PerkinElmer), fitted with a Thermogreen LB-2 predrilled septum, and a 1 mm liner. A flow of helium of 1 mL/min was used as the carrier gas. Splitless mode was applied for injection. A nonpolar capillary column, coated with 95% dimethyl-/5% diphenyl-siloxane (30 m × 0.25 mm × 0.25 μm film thickness, Equity 5, Supelco) was used. The oven temperature program started at 40 °C, held for 2 min, followed by an increase of 6 °C/min to the final temperature of 200 °C, held for 8 min. A cooldown ramp was added, decreasing the temperature to 40 °C at 20 °C/min, held for 4 min. The total run lasted 43 min. The electron ionization Clarus 500 MS (PerkinElmer) was equipped with a quadrupole, and set to scan for mass-to-charge ratios between 41–300 *m/z* after a 2 min delay. These scanning parameters were set after a refining process aimed to reduce baseline noise to a minimum. Before each sample was analyzed, the fiber was conditioned 1 min at 250 °C in the injection port of the GC–MS; then a blank run, in which nothing

was injected, was performed, to ensure the GC column was clean. Samples were analyzed in random order to reduce the chance of artificially creating a batch effect. Blank samples (i.e. five blank fibers, three empty vials, and two vials containing an unused swab) were added to the pool of samples to analyze, in order to identify extraneous contaminant compounds in the samples.

For each chromatogram, automatic peak detection, integration, and tentative identification using the National Institute of Standards and Technology (NIST) mass spectral library (Shen et al. 2014), was performed in ChemStation (Agilent, Santa Clara, CA, USA). Only peaks with a minimum area of 1% of that of the largest genuine peak were selected, in order to limit the inclusion of background noise (Drea et al. 2013). In addition, 90 peaks found in at least 1 of the blank samples were excluded from the pool of peaks that were further analyzed. Furthermore, 63 peaks for which visual inspection of the mass spectra combined with NIST results showed them to be likely contaminants (e.g. phthalates, siloxanes), were also excluded. Relative peak abundance for the 127 remaining peaks was calculated to represent relative proportion within each sample, allowing us to account for varying intensities of the collected samples, both in terms of the amount of the secretion collected, and the concentration of the different components (relative peak abundance = peak area/sum of peak areas in sample × 100, excluding contaminants). These values were used to estimate chemical distances and select peaks with the highest contribution to the overall dissimilarity between categories of species, sex and reproductive status. Careful visual comparison of the peaks' mass spectra made it possible to determine whether peaks of similar retention times represented the same or different compounds. The identities of 13 compounds were further confirmed by comparison of their retention times with those of commercially obtained compounds, analyzed under identical conditions. Since authentic compounds and corresponding commercially obtained compounds were not co-injected, their retention times usually differed slightly, as retention times may vary between GC–MS runs. Hence, compound identity was considered validated if the mass spectra of the compound in the sample and that of the commercially obtained compound were closely matched, and the retention time of the compound in the sample fell within the width of the genuine compound's peak at mid-height; this was visually assessed. In some cases, identification was less certain, with commercially obtained samples eluting a short time after the corresponding compounds in the samples. In such instances, we suggested that the compounds in the secretion were branched-chain variants of the same molecular weight as the commercially obtained compounds.

Statistical analyses

All statistical analyses were performed in R v.4.0.2 operated in RStudio (R Core Team 2020). A generalized linear mixed model adapted to zero-inflation (glmmTMB function in R package *glmmTMB* v.1.0.2.1; Brooks et al. 2017) with *Poisson* family was fitted to assess the relationship between hourly scent-marking frequency, and the species, sex and reproductive status of the signaler, as well as scent gland used. The use of sternal gland for scent-marking was very low ($n = 12$) compared with anogenital ($n = 617$) and suprapubic ($n = 78$) glands, therefore these scent-marking events were not included in the model. Hourly scent-marking frequencies were zero inflated, and therefore required a zero-inflated model (ziformula = ~1) for this response variable. We included species, sex, reproductive status, and scent gland use as fixed effects; and individual (nested into group), day of observation (nested into group) and observation bout (nested into day and group) as random effects. Inspection of model

residuals, produced using the simulateResiduals and testResiduals functions in R package *DHARMa* v.0.3.3.0 (Hartig 2020), did not reveal any obvious heteroscedasticity or overdispersion in the data.

Variation in chemical composition between groups of samples at the levels of species, sex, and reproductive status was assessed using Nonmetric Multidimensional Scaling (NMDS), which allowed quantification and graphical visualization of sample chemical composition, followed by Permutational Multivariate Analysis of Variance Using Distance Matrices (PerMANOVA), computed using the R package *vegan* v.2.5–7 (Oksanen et al. 2020). This package was created for the multivariate analysis of ecological communities, providing ordination and diversity analysis methods to explore patterns of presence/absence, or abundance, of animal and vegetal species within an ecological community. It has notably been employed in semiochemical studies of Australian sea lions, *Neophoca cinerea* (Wierucka et al. 2019), and meerkats, *Suricata suricatta* (Leclaire et al. 2017). The first step in assessing sample chemical diversity was to compute a distance matrix of samples and their respective compound composition, using the vegdist function with the Bray-Curtis dissimilarity index. This index measured chemical dissimilarity between every pair of samples based on the $\log(x + 1)$ transformed relative peak abundances. Then, two-dimensional NMDS coordinates were calculated from the values of the Bray–Curtis dissimilarity index in the distance matrix, with the metaMDS function. These two coordinates allowed for the visualization of dissimilarity between groups of samples. The stress factor measured the goodness-of-fit between predicted and observed values (similar to the R^2 value in a regression), and was considered a good fit when stress ≤0.2. A Multifactorial PerMANOVA, a nonparametric method fitting a linear model to the distance matrix, was then carried out to assess the relative effect of species, sex, and reproductive status on sample chemical diversity. We used the adonis2 function with 999 permutations and the Bray–Curtis dissimilarity index, which allowed the effect of one predictor to be assessed while accounting for the effects of other predictors (arg = "margins" argument). We included the effects of species, sex, and reproductive status, as well as individual (nested into group) as a way to control for repeated sampling of the same individuals. We did not include interactions terms as these were found to be non-significant in a full model. Unlike most statistical models, the only statistical assumption of PerMANOVA is to ensure multivariate homogeneity of variance within each group tested. This assumption was verified using the permutation test for homogeneity of multivariate dispersion (permutest function using 999 permutations), on the measure of group multivariate homogeneity of variance computed using the betadisper function. Finally, a similarity percentage analysis (SIMPER), using the simper function in *vegan* on the distance matrix was used to determine the relative contribution of each peak to the chemical dissimilarity between categories of species, sex, and reproductive status. Peaks were ranked by their contribution to the overall dissimilarity, calculated as the mean contribution score for species, sex and reproductive status categories. We selected as "compounds of interest" the peaks of the highest rank, that is with a mean contribution ≥0.01. The identity of these compounds of interest ($n = 41$) and their presence in the different categories of samples were further investigated.

Results

Differences in scent-marking frequency

We recorded a total of 707 individual scent-marking events across the 18 animals from the 4 groups of cotton-top and emperor tamarins

studied. The number of scent-marking events recorded per hour ranged from 0–19 per group, and from 0–18 per individual. Cotton-top tamarins scent-marked 2.19 ± 3.15 times per hour of observation (mean ± standard deviation [SD]; $n = 10$ individuals), emperor tamarins 1.34 ± 1.61 times per hour ($n = 8$); these rates did not statistically differ (Table 2). In both species, females marked significantly more (cotton-top: 3.56 ± 3.83 marks per hour, $n = 4$; emperor: 1.89 ± 1.89 marks per hour, $n = 4$) than males (cotton-top: 0.83 ± 1.21 marks per hour, $n = 6$; emperor: 0.79 ± 1.01 marks per hour, $n = 4$); and reproductive individuals marked significantly more (cotton-top: 3.24 ± 3.96 marks per hour, $n = 4$; emperor: 2.01 ± 1.88 marks per hour, $n = 4$) than non-reproductive ones (juveniles and subordinates; cotton-top: 1.15 ± 1.40 marks per hour, $n = 6$; emperor: 0.67 ± 0.88 marks per hour, $n = 4$; Table 2). The anogenital scent gland was used the most for scent-marking (cotton-top: 1.92 ± 3.10 marks per hour; emperor: 1.17 ± 1.56 marks per hour), followed by the suprapubic gland (cotton-top: 0.24 ± 0.58 marks per hour; emperor: 0.15 ± 0.43 marks per hour; Table 2). The sternal gland was used the least (cotton-top: 0.04 ± 0.20 marks per hour; emperor: 0.02 ± 0.14 marks per hour) and was not included in the linear model.

Differences in scent mark chemical composition

Analysis by SPME–GC-MS of the 34 anogenital scent mark samples collected from cotton-top and emperor tamarins revealed a total of 127 different volatile compounds (Supplementary Table S1). The number of compounds in each sample ranged from 5–34 (mean ± SD = 18.24 ± 7.71 compounds). While 34.6% (n = 44) of compounds were unique to a scent mark sample, the remainder were common to at least two samples. Only 6.3% ($n = 8$) of the compounds were present in more than half of the samples. Supplementary Figure S1 shows typical examples of gas chromatograms obtained from scent mark swabs of reproductive female cotton-top and emperor tamarins.

Sample chemical composition differed between cotton-top and emperor tamarins (PerMANOVA: $F_{1,20} = 8.10$, $R^2 = 0.16$, $P < 0.01$); the species appear well separated on the NMDS plot (Figure 1a). Differences in chemical composition were also significant for sex ($F_{1,20} = 2.27$, $R^2 = 0.05$, $P = 0.01$) and reproductive status ($F_{1,20} = 2.24$, $R^2 = 0.04$, $P = 0.01$), though to a lesser level, as indicated by the minor discrimination observed for sex and reproductive status on Figure 1b and c, respectively.

Although the automated NIST mass spectral library provided putative identities for all 127 compounds retrieved from the samples, we focused on a subset of 41 compounds of highest relative contribution to the dissimilarity between categories of species, sex and reproductive status, for which the NIST library identity was individually verified by visual inspection of the peaks' mass spectra and retention times. These compounds are thereafter referred to as compounds of interest, listed in Table 3. Whenever possible, the identity of a compound was confirmed, or refuted, by comparison of its retention time with that of the commercially obtained compound analyzed under the same conditions. As a result, we positively identified 13 compounds (marked with an asterisk in Table 3). The majority of the compounds of interest retrieved from the samples were hydrocarbons (alkanes, cycloalkanes and aromatic hydrocarbons, 24.4%), alcohols (22%), and aldehydes (17.1%). Over 30% of these compounds contained an aromatic group (e.g. benzaldehyde [#10]) or a furan ring (e.g. 2-furanmethanol [#05]); the rest were aliphatic, either straight (e.g. hexanal [#02]), branched (e.g. 3-methylbutanoic acid [#04]), or cyclic compounds (e.g. cyclodecane [#28]).

The majority of compounds of interest were common to both tamarin species ($n = 29$; 70.7%); four compounds were unique to cotton-top tamarins; and seven to emperor tamarins (Table 3). In both species, a number of compounds of interest were shared between males and females (cotton-top: $n = 23$, 67.6%; emperor: $n = 28$, 75.7%), and between reproductive and non-reproductive individuals (cotton-top: $n = 26$, 76.5%; emperor: $n = 29$, 78.4%); a single compound, longifolene [#32], was only found in female emperor tamarins, while no compound was specific to female cotton-top tamarins or to males of either species (Table 3).

Among the 41 compounds of interest with highest contribution to the overall sample chemical dissimilarity, the 10 compounds responsible for most chemical dissimilarity between cotton-top and emperor tamarins were *p*-cresol [#20], 2-methoxyphenol [#21], cyclodecane [#28], *trans*-1-methyl-4-(1-methylethyl)cyclohexanol [#25], 4-methoxybenzaldehyde [#27], diethylene glycol dibutyl ether [#34], benzaldehyde [#10], 2-furanmethanol [#05], hexanal [#02], and 1,2,4-trimethylbenzene [#13]; those between males and females were 4-methoxybenzaldehyde [#27], *p*-cresol [#20], cyclodecane [#28], benzaldehyde [#10], diethylene glycol dibutyl ether [#34], 2-methoxyphenol [#21], 1,2,4-trimethylbenzene [#13], 2-furanmethanol [#05], *trans*-1-methyl-4-(1-methylethyl)cyclohexanol [#25], and hexanal [#02]; and those between reproductive and non-reproductive individuals were *p*-cresol [#20], cyclodecane [#28], 4-methoxybenzaldehyde [#27], diethylene glycol dibutyl ether [#34], benzaldehyde [#10], 2-furanmethanol [#05], 2-methoxyphenol [#21], *trans*-1-methyl-4-(1-methylethyl)cyclohexanol [#25], cyclododecane [#36], and hexanal [#02] (Figure 2).

Table 2. Results of generalized linear mixed model (*Poisson* family) testing the difference in hourly scent-marking frequency between species, sex, reproductive status and scent gland use. *P*-values are significant at $P \leq 0.05$ (in bold). SE = standard error of the mean; SD = standard deviation

Fixed effects	Paired comparisons†	Estimate (± SE)	Z-statistic	P
(Intercept)		−1.24 (± 0.43)	−2.90	**< 0.01**
Species	Cotton-top–Emperor	0.10 (± 0.41)	0.24	0.81
Sex	Female–Male	−0.95 (± 0.41)	−2.33	**0.02**
Repro. status	Non-reproductive–Reproductive	1.40 (± 0.41)	3.34	**< 0.01**
Scent gland	Anogenital–Suprapubic	−2.02 (± 0.13)	−15.93	**< 0.01**
Random effects		Variance (± SD)		
Group: individual		0.63 (± 0.80)		
Group: day		0.02 (± 0.14)		
Group: day: bout		0.20 (± 0.44)		

†The first level mentioned is the reference level in each paired comparison.
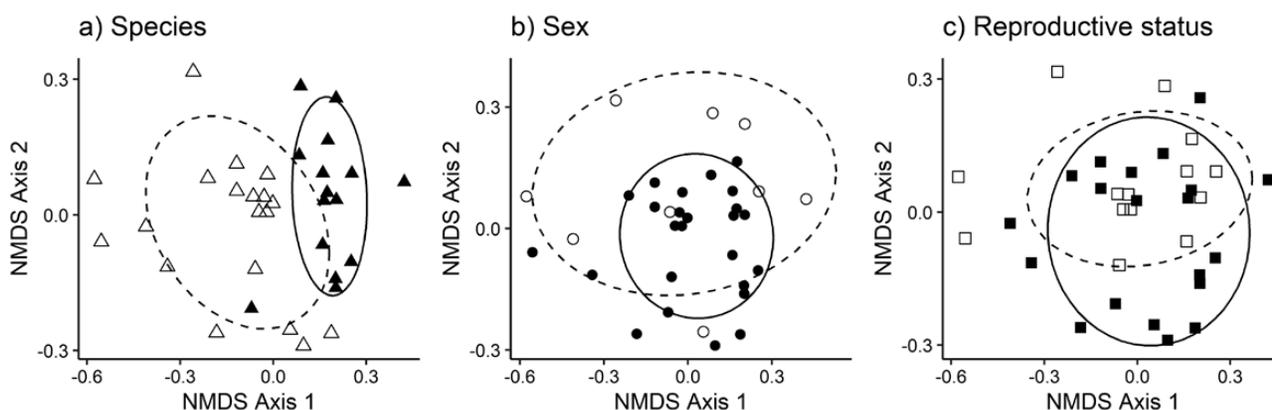
Figure 1. Nonmetric multidimensional scaling (NMDS) visualization of differences in sample chemical composition between (**a**) cotton-top tamarins (▲, solid line), and emperor tamarins (△, dashed line); (**b**) females (●, solid line) and males (○, dashed line), and (**c**) reproductive (■, solid line) and non-reproductive (□, dashed line) individuals. Scaling was based on Bray–Curtis dissimilarities calculated using standardized relative abundance of the 127 peaks retrieved from the samples (stress = 0.19). Points in close proximity indicate a higher chemical similarity of samples. Ellipses represent the 75% confidence interval for categories of samples.

Discussion

Our results revealed differences in scent-marking behavior and semiochemistry of captive cotton-top and emperor tamarins at the levels of species, sex and reproductive status. They support the idea that olfactory communication plays an important role in the advertisement of species, sex and reproductive status in this taxon. We further identified 41 compounds of possible semiochemical importance in tamarins. Nevertheless, the limited number of scent mark samples collected restricted interpretations of the chemical results obtained. Moreover, the number of individuals included in the present study was relatively small; the two species studied were observed at three different sites, and the 4 tamarin groups differed in size and composition, which did not permit discrimination between species, site and group differences.

We observed no statistical difference in scent-marking activity between cotton-top and emperor tamarins, which was expected given the similar environmental conditions of the two species in this captive study. Other studies on wild and zoo-kept callitrichids have generally found similar lower hourly frequencies; whereas a much higher scent-marking rate has been reported in laboratory-kept callitrichids (Table 4). This difference may stem from the fact that visibility of the animals' behavior is poorer in wild conditions and in large enclosures, in which a number of the deposited scent-marks may fail to be recorded. Nevertheless, the chemical composition of their anogenital scent marks differed, suggesting species-specific chemical communication in callitrichids. Other semiochemical studies have found similar results (e.g. in glandular secretions of owl monkeys, Spence-Aizenberg et al. 2018; sympatric Siberian weasels and steppe polecats, Zhang et al. 2002; and several large felines, Soini et al. 2012; in the urine of brown lemurs, *Eulemur* spp., DelBarco-Trillo et al. 2011; and several *Phodopus* hamster species, Soini et al. 2005).

Overall, tamarin females scent-marked more frequently than males, and reproductive individuals more than non-reproductive ones. Similar results have been found in tamarins in captivity (e.g. in cotton-top tamarins and saddleback tamarins, *Saguinus fuscicollis*, French and Snowdon 1981; and red-bellied tamarins, Coates and Poole 1983; Smith and Gordon 2002), as well as in the wild (e.g. in moustached tamarins, *S. mystax*, Heymann 1998; and golden lion tamarins, Miller et al. 2003). In addition, we found chemical differences in scent mark samples at the levels of sex and reproductive

status, which is consistent with a role of olfactory communication in mate choice, intrasexual competition, dominance and/or reproductive suppression in callitrichids. Other studies have found semiochemicals indicative of sex (e.g. in glandular secretions of owl monkeys, MacDonald et al. 2008; mandrills, Setchell et al. 2010; giant pandas, Hagey and MacDonald 2003; Yuan et al. 2004; banded mongooses, *Mungos mungo*, Jordan et al. 2011; and brown bears, *Ursus arctos*, Rosell et al. 2011; and urine of lions, *Panthera leo*, Andersen and Vulpius 1999; and binturongs, *Arctictis binturong*, Greene et al. 2016b); as well as of reproductive state (e.g. in secretions of female Coquerel's sifakas, Greene and Drea 2014; Alpine marmots, *Marmota marmota*, Zidat et al. 2018; in urine of house mice, *Mus musculus*, Andreolini et al. 1987; and Eurasian lynxes, *Lynx lynx*, Vogt et al. 2016.; and feces of white rhinos, *Ceratotherium simum*, Marneweck et al. 2017). Anogenital and suprapubic scent glands of callitrichids are larger in females than males (first reported in Perkins 1975; in cotton-top tamarins, French and Cleveland 1984; and saddleback tamarins, Zeller et al. 1988). Unlike males, female reproductive state varies cyclically with ovulation. While many female primates provide visual and/or acoustic cues of ovulation (e.g. sexual swellings in female mandrills, Setchell 2016; mating calls in female Barbary macaques, *Macaca sylvanus*, Pfefferle et al. 2008), in female callitrichids ovulation is thought to be concealed (Dixson 2012). Although female callitrichids engage in sexual behavior throughout their estrous cycle (ca. 23 days in tamarins; French et al. 1984), several studies have shown an increase of male sexual activity in the female periovulatory period (Smith and Abbott 1998; Ziegler et al. 2005). Female callitrichids are therefore assumed to communicate their reproductive state via odor cues (e.g. in cotton-top tamarins, Ziegler et al. 1993; common marmosets, Kücklich et al. 2019; and pygmy marmosets, *Cebuella pygmaea*, Converse et al. 1995). Although our results indicate a global role of olfactory cues in signaling female reproductive status, we were not able to measure individual differences within the female estrous cycle. This could only be reliably measured through hormone analyses, which was beyond the scope of this study.

Tamarins of both species preferentially used their anogenital scent gland when scent-marking, followed by the suprapubic gland. This was also found in another study on cotton-top tamarins (French and Cleveland 1984), as well as in other tamarin species (reviewed in Heymann 2001). The scent glands are very similar in their histology

Table 3. Subset of 41 compounds of interest retrieved from the samples and their presence in the different sample categories: cotton-top and emperor tamarins, male and female, reproductive (R) and non-reproductive (NR). Putative identity of the compounds was established by mass spectral library search, then verified by visual inspection of the mass spectra and retention times (RT, in min). Identity of the 13 compounds marked with an asterisk (*) was confirmed by comparison of their retention times with those of commercially obtained compounds. SD = standard deviation

#	Mean RT ± SD	Candidate compound identity	Number of samples containing compound (and % in each category)								
			Total (n = 34)	Cotton-top				Emperor			
				R♂ (n = 2)	R♀ (n = 7)	NR♂ (n = 2)	NR♀ (n = 4)	R♂ (n = 2)	R♀ (n = 9)	NR♂ (n = 3)	NR♀ (n = 5)
01	4.68 ± 0.05	Propane-1,2-diol	3 (8.8%)	–	–	–	–	–	1 (11.1%)	2 (66.7%)	–
02	6.14 ± 0.02	Hexanal*	25 (73.5%)	1 (50%)	4 (57.1%)	2 (100%)	3 (75%)	2 (100%)	8 (88.9%)	1 (33.3%)	4 (80%)
03	6.23 ± 0.02	Furfural	9 (26.5%)	–	1 (14.3%)	–	1 (25%)	–	4 (44.4%)	1 (33.3%)	1 (20%)
04	8.06 ± 0.11	3-Methylbutanoic acid	7 (20.6%)	1 (50%)	3 (42.9%)	1 (50%)	–	–	1 (11.1%)	1 (33.3%)	–
05	8.04 ± 0.02	2-Furanmethanol*	17 (50%)	–	3 (42.9%)	1 (50%)	3 (75%)	1 (50%)	5 (55.6%)	2 (66.7%)	2 (40%)
06	9.15 ± 0.02	Methylcycloheptanone	6 (17.6%)	–	1 (14.3%)	–	1 (25%)	1 (50%)	2 (22.2%)	–	1 (20%)
07	9.33 ± 0.02	Heptanal*	21 (61.8%)	1 (50%)	4 (57.1%)	2 (100%)	3 (75%)	1 (50%)	6 (66.7%)	1 (33.3%)	3 (60%)
08	9.80 ± 0.01	Anisole*	6 (17.6%)	–	3 (42.9%)	–	3 (75%)	–	–	–	–
09	10.25 ± 0.02	α-Pinene	10 (29.4%)	1 (50%)	3 (42.9%)	1 (50%)	4 (100%)	–	–	1 (33.3%)	–
10	11.14 ± 0.03	Benzaldehyde*	28 (82.4%)	1 (50%)	6 (85.7%)	2 (100%)	4 (100%)	2 (100%)	7 (77.8%)	1 (33.3%)	5 (100%)
11	11.33 ± 0.00	1,3,5-Trimethylbenzene*	3 (8.8%)	–	–	2 (100%)	1 (25%)	–	–	–	–
12	11.76 ± 0.04	Unknown compound 1	16 (47.1%)	1 (50%)	3 (42.9%)	1 (50%)	2 (50%)	–	4 (44.4%)	2 (66.7%)	3 (60%)
13	12.08 ± 0.01	1,2,4-Trimethylbenzene*	7 (20.6%)	2 (100%)	1 (14.3%)	2 (100%)	2 (50%)	–	–	–	–
14	12.93 ± 0.03	1,2,3-Trimethylbenzene	6 (17.6%)	–	–	2 (100%)	4 (100%)	–	–	–	–
15	13.18 ± 0.01	Butanamide	6 (17.6%)	–	–	–	–	–	2 (22.2%)	1 (33.3%)	3 (60%)
16	13.56 ± 0.04	Benzeneacetaldehyde*	11 (32.4%)	1 (50%)	1 (14.3%)	2 (100%)	1 (25%)	1 (50%)	2 (22.2%)	1 (33.3%)	2 (40%)
17	13.70 ± 0.05	N,N-Dimethylbenzylamine	8 (23.5%)	–	1 (14.3%)	2 (100%)	1 (25%)	–	1 (11.1%)	–	3 (60%)
18	14.21 ± 0.04	Acetophenone*	20 (58.8%)	2 (100%)	2 (28.6%)	–	1 (25%)	1 (50%)	7 (77.8%)	3 (100%)	4 (80%)
19	14.39 ± 0.03	1-Octanol	6 (17.6%)	–	2 (28.6%)	–	1 (25%)	–	2 (22.2%)	–	1 (20%)
20	14.57 ± 0.05	p-Cresol*	22 (64.7%)	2 (100%)	7 (100%)	2 (100%)	4 (100%)	–	3 (33.3%)	1 (33.3%)	3 (60%)

Table 3. Continued

#	Mean RT ± SD	Candidate compound identity	Number of samples containing compound (and % in each category)									
			Total (n = 34)	Cotton-top				Emperor				
				R♂ (n = 2)	R♀ (n = 7)	NR♂ (n = 2)	NR♀ (n = 4)	R♂ (n = 2)	R♀ (n = 9)	NR♂ (n = 3)	NR♀ (n = 5)
21	14.85 ± 0.04	2-Methoxyphenol	19 (55.9%)	1 (50%)	6 (85.7%)	1 (50%)	4 (100%)	–	3 (33.3%)	1 (33.3%)	3 (60%)
22	15.07 ± 0.03	3,7-Dimethyloctan-3-ol	7 (20.6%)	1 (50%)	1 (14.3%)	–	–	2 (100%)	3 (33.3%)	–	–
23	16.13 ± 0.04	Dimethyl pentanedioate*	6 (17.6%)	2 (100%)	–	1 (50%)	–	1 (50%)	2 (22.2%)	–	–
24	16.28 ± 0.04	Unknown C9 branched alcohol	7 (20.6%)	–	–	–	–	1 (50%)	4 (44.4%)	–	2 (40%)
25	16.62 ± 0.04	trans-1-Methyl-4-(1-methylethyl) cyclohexanol	15 (44.1%)	–	–	–	–	1 (50%)	6 (66.7%)	3 (100%)	5 (100%)
26	16.98 ± 0.04	1-Nonanol*	11 (32.4%)	1 (50%)	3 (42.9%)	1 (50%)	1 (25%)	1 (50%)	1 (11.1%)	–	3 (60%)
27	19.08 ± 0.06	4-Methoxybenzaldehyde*	32 (94.1%)	2 (100%)	7 (100%)	2 (100%)	4 (100%)	2 (100%)	9 (100%)	2 (66.7%)	4 (80%)
28	19.43 ± 0.03	Cyclodecane	22 (64.7%)	–	6 (85.7%)	2 (100%)	3 (75%)	1 (50%)	5 (55.6%)	1 (33.3%)	4 (80%)
29	19.93 ± 0.05	Unknown C13 branched alkane	12 (35.3%)	–	1 (14.3%)	–	–	1 (50%)	5 (55.6%)	2 (66.7%)	3 (60%)
30	21.25 ± 0.01	Glycerol 1,2-diacetate	2 (5.9%)	–	1 (14.3%)	–	–	–	1 (11.1%)	–	–
31	22.49 ± 0.01	Do- or tri-decanal	5 (14.7%)	–	2 (28.6%)	1 (50%)	1 (25%)	–	1 (11.1%)	–	–
32	22.49 ± 0.00	Longifolene	5 (14.7%)	–	–	–	–	–	2 (22.2%)	–	3 (100%)
33	22.74 ± 0.00	Unknown compound 2	3 (8.8%)	2 (100%)	–	–	1 (25%)	–	–	–	–
34	23.09 ± 0.05	Diethylene glycol dibutyl ether	10 (29.4%)	–	–	–	–	–	3 (33.3%)	3 (100%)	4 (80%)
35	23.41 ± 0.07	6,10-Dimethyl-5,9-undecadien-2-one	11 (32.4%)	–	1 (14.3%)	–	–	1 (50%)	7 (77.8%)	2 (66.7%)	–
36	23.85 ± 0.07	Cyclododecane	16 (47.1%)	–	3 (42.9%)	–	1 (25%)	1 (50%)	3 (33.3%)	3 (100%)	5 (100%)
37	24.94 ± 0.06	Myristicin	12 (35.3%)	1 (50%)	1 (14.3%)	2 (100%)	2 (50%)	–	1 (11.1%)	2 (66.7%)	3 (60%)
38	25.24 ± 0.01	2-Methyldecyl propanoate	5 (14.7%)	–	2 (28.6%)	–	2 (50%)	–	1 (11.1%)	–	–
39	25.68 ± 0.07	Unknown C16 branched alkane 1	5 (14.7%)	–	–	–	–	1 (50%)	1 (11.1%)	2 (66.7%)	1 (20%)
40	26.26 ± 0.07	Unknown C16 branched alkane 2	16 (47.1%)	1 (50%)	3 (42.9%)	–	–	1 (50%)	4 (44.4%)	3 (100%)	4 (80%)
41	30.01 ± 0.07	Unknown C18 branched alkane	5 (14.7%)	–	–	–	–	–	–	3 (100%)	2 (40%)
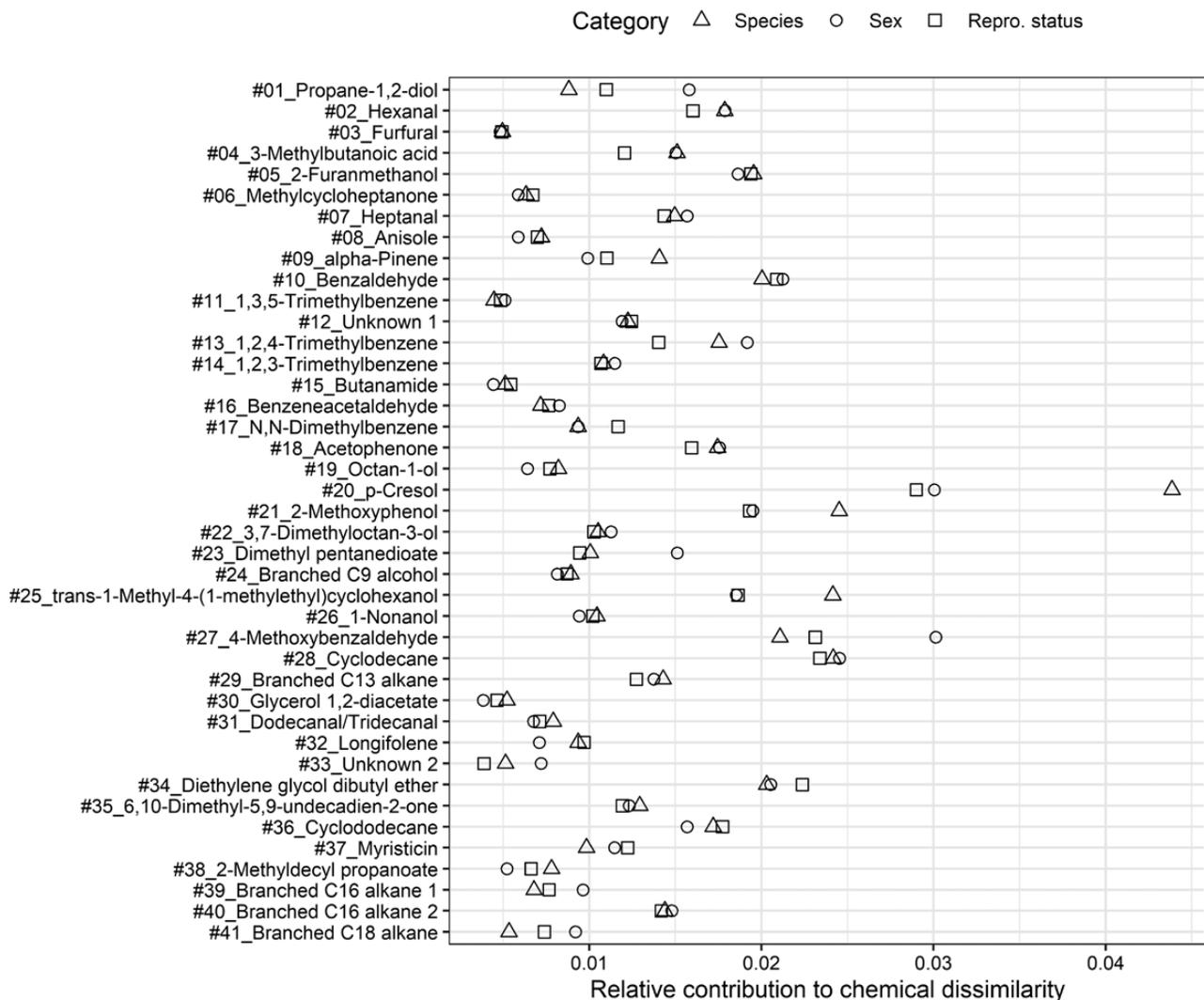
Figure 2. Relative contribution to sample chemical dissimilarity for species, sex and reproductive status, calculated from Bray–Curtis dissimilarities between pairs of samples and compounds of interest.

Table 4. Hourly scent-marking rates reported in various wild and captive callitrichid species

Study condition	Species	Hourly scent-marking rate (± SD when given)	References
Wild	Moustached tamarins, *Saguinus mystax*	0.79; 0.52	Smith 1997; Heymann 2001
	Geoffroy's saddleback tamarins, *S. fuscicollis*	24.68; 5.59	Smith 1997; Heymann 2001
	Common marmosets, *Callithrix jacchus*	2.06	Lazaro-Perea et al. 1999
	Golden lion tamarins, *Leontopithecus rosalia*	2.30 ± 0.41	Miller et al. 2003
Captive (zoo)	Cotton-top tamarins, *S. oedipus*	2.19 ± 3.15	This study
	Bearded emperor tamarins, *S. imperator subgrisescens*	1.34 ± 1.61	This study
	Red-bellied tamarins, *Saguinus labiatus*	1.88	Smith and Gordon 2002
Captive (laboratory)	Cotton-top tamarins	25	French and Snowdon 1981
	Red-bellied tamarins (females only)	13	Coates and Poole 1983

(Perkins 1966, 1975; Fontani et al. 2014). Nonetheless, the chemicals secreted by each gland might vary in their identity and/or concentration, potentially leading to the production of different scent signals and cues. A handful of studies have identified differences in the chemical composition of primate scent glands, notably between anogenital, suprapubic, and sternal glands of wild sympatric emperor tamarins and Weddell's saddleback tamarins, *Leontocebus weddelli*

(Poirier et al. 2021), genital and brachial glands of ring-tailed lemurs (Scordato et al. 2007), and subcaudal and pectoral glands of owl monkeys (Spence-Aizenberg et al. 2018). Unfortunately, all samples collected for this project originated from anogenital scent marks, preventing a comparison at the chemical level.

The total number of compounds found in samples from both tamarin species (*n* = 127) was in the range of previous findings

in primate semiochemistry: Smith et al. (2001) found 162 compounds in scent marks of female common marmosets; Greene and Drea (2014) detected 252 compounds in the genital secretions of Coquerel's sifakas; MacDonald et al. (2008) found 300 compounds in the subcaudal gland secretions of owl monkeys; but Setchell et al. (2010) found only 47 compounds in 88 swabs of mandrill sternal gland secretions; and Delbarco-Trillo et al. (2011) retrieved 74 volatiles from the urine of twelve species of brown lemurs. Different methods for chemical sample collection, extraction and analyses are likely to yield different results (Drea et al. 2013; Kücklich et al. 2017), which may have contributed to the differences observed between studies. The sample extraction technique employed, headspace SPME, was selective for the more volatile components of the samples. Indeed, the compound of highest molecular weight identified in the present study was a branched C18 alkane with a molecular weight of 254.5 g/mol and a boiling point between 302–317 °C, while other studies using different methods retrieved compounds of much higher molecular weight (e.g. squalene, with a molecular weight of 411 g/mol, retrieved from mandrill scent gland secretions; Setchell et al. 2010). Moreover, the most volatile components could have dissipated soon after being secreted, before sampling. Therefore, the true *odor bouquet* conveyed in individual callitrichid scent marks is likely to be more complex than the assemblage of compounds retrieved in this study.

The compounds of interest identified in this study ($n = 41$) were mainly hydrocarbons, alcohols, aldehydes, and ketones, sometimes containing an aromatic group, many of which have been reported in secretions or urine of other primates and other mammalian taxa, mainly carnivores, rodents and artiodactyls (Supplementary Table S2). Hexanal [#02], 3-methylbutanoic acid [#04], heptanal [#07], benzaldehyde [#10], acetophenone [#18], and *p*-cresol [#20] were particularly widespread compounds, reported in over 27 different mammalian taxa (Supplementary Table S2). Because of the very limited number of samples in this study, it is not possible to come to specific conclusions as to the chemical differences between species, sex and reproductive status. However, we note that a few of the identified compounds appeared to be specific to single sample categories. Notably, 1,3,5-trimethylbenzene [#11], 1,2,3-trimethylbenzene [#13] and 1,2,4-trimethylbenzene [#14] were unique to cotton-top tamarins; propane-1,2-diol [#01], butanamide [#15], an unknown C9 branched alcohol [#24], *trans*-1-methyl-4-(1-methylethyl) cyclohexanol [#25], longifolene [#32], diethylene glycol dibutyl ether [#34], an unknown C16 branched alkane [#39] and an unknown C18 branched alkane [#41] to emperor tamarins. A single compound, longifolene [#32], was uniquely found in female emperor tamarins. Further work is, however, required, including more animals and repeated sampling of individuals, before any firm conclusions can be drawn about the semiochemical role of these compounds at the level of species, sex, or reproductive status.

Several of the compounds found may have not been directly produced by the animals. In particular, anisole [#08], α-pinene [#09], *p*-cresol [#20], 2-methoxyphenol [#21], and longifolene [#32] are definitely of non-mammalian origin, because their metabolic pathway only exists in plants, fungi, and bacteria (Charpentier et al. 2012). These compounds may originate from diet, in which case they are secreted as unmetabolized compounds. Ferkin et al. (1997) experimentally demonstrated that differences in diet affected the attractiveness of meadow voles, *Microtus pennsylvaticus*, to the other sex, although the exact mechanism by which this happened was not elucidated. Moreover, commensal bacteria present in the scent glands or on the skin may take an active part in the chemical composition

of the secretions (Theis et al. 2013; Leclaire et al. 2017). In addition, some compounds of plant origin may have been incorporated into the swabs during sampling and correspond to the odor of the substrate branches themselves, or to contamination of the surface swabbed by food remains and/or excrement. Nevertheless, callitrichids, like other animals, may very well use such extraneous compounds as semiochemicals, even if they do not directly produce them.

Our study revealed differences in scent-marking behavior and semiochemistry of captive cotton-top and emperor tamarins at the levels of species, sex and reproductive status. We identified 41 compounds of possible semiochemical importance in tamarins. Further research is necessary to establish their semiochemical role, if any. In particular, combining chemical analyses with behavioral bioassays, in which odors are experimentally presented to target animals, may be a good way to reach further into understanding the variable function of the diverse forms of chemical cues used in olfactory communication (e.g. in common marmosets, Smith et al. 1997; Smith and Abbott 1998; in ring-tailed lemurs, Greene and Drea 2014; Shirasu et al. 2020). Moreover, comparing the present study on ecologically similar yet geographically distinct tamarin species, to studies of co-occurring species which do not form mixed-species groups, as well as co-occurring species which form mixed-species groups, would provide a more comprehensive model system to demonstrate the role of chemosignaling in species recognition mechanisms. Since our results were collected non-invasively by swabbing deposited scent marks, they constitute a promising method for the study of animal olfactory communication, applicable to field settings and for the study of elusive animals.

Conflict of interest

Funding

Data Availability

Supplementary material can be accessed at http://www.chemse.oxfordjournals.org. The data and R code supporting this article are available at https://github.com/AlicePoirier/Poirier-et-al_ChemSenses_data_Feb2021.

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