Relatedness communicated in lemur scent

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Abstract

Lemurs are the most olfactory-oriented of primates, yet there is still only a basic level of understanding of what their scent marks communicate. We analyzed scent secretions from Milne-Edwards' sifakas (*Propithecus edwardsi*) collected in their natural habitat of Ranomafana National Park, Madagascar. We sought to test whether the scent mark could signal genetic relatedness in addition to species, sex, season, and individuality. We not only found correlations ($r^2 = 0.38$, $P = 0.017$) between the total olfactory fingerprint and genetic relatedness, but also between relatedness and specific components of the odor, despite the complex environmental signals from differences in diet and behaviour in a natural setting. To the best of our knowledge, this is the first demonstration of an association between genetic relatedness and chemical communication in a wild primate population. Furthermore, we found a variety of compounds that were specific to each sex and each sampling period. This research shows that scent marks could act as a remote signal to avoid inbreeding, optimize mating opportunities, and potentially aid kin selection.

Key words

chemical communication, gas chromatography-mass spectrometry (GC-MS), kin recognition, Madagascar, Strepsirrhini

Introduction

Relatedness can be an important factor in mate choice, as it defines the probability that two individuals share a deleterious recessive allele at a given locus, and thus affects the likelihood of inbreeding depression in their offspring (Charlesworth and Charlesworth 1987; Billing et al. 2012). If individuals are capable of kin recognition (Tang-Martinez 2001; Penn and Frommen 2010), they can avoid inbreeding depression by optimizing mate choice, as well as increase inclusive fitness by directing nepotism toward relatives (e.g. Van Horn et al. 2004; Thorington and Weigl 2011). Odor has been shown to be the cue used to recognize relatives in many species, although there are few cases where this has been demonstrated in natural settings (e.g., Thom et al. 2008; Leclaire et al. 2012). For example, the major histocompatibility complex (MHC) of the vertebrate immune system creates byproducts that can be detected through olfaction (Schwensow et al. 2008; Ruff et al. 2012).

An extensive body of research has begun to accumulate about the use of olfactory communication by mammals in general (Gosling and Roberts 2001) and primates in particular (Evans 1980; Price and Feistner 1994; Ramsay and Giller 1996; Charpentier et al. 2008; Charpentier et al. 2010; Setchell et al. 2010; Setchell et al. 2011), advanced by the fact that most primate species are highly social and well-studied and captive colonies are available for experimental tests. Researchers have studied prosimian olfactory communication in particular as they rely more on scent than most other primates (Fisher et al. 2003; Dapporto 2008; Charpentier et al. 2010). Prior studies of lemurs in the wild concentrated on the characteristics of the animal, the context of scent-marking, or the response to the scent mark

(Harrington 1977; Evans 1980; Kappeler 1993; Price and Feistner 1994; Ramsay and Giller 1996; Pochron et al. 2005a; Pochron et al. 2005b). More recent work has tried to illuminate the molecular details of olfactory communication, with Drea and colleagues (Charpentier et al. 2008; Boulet et al. 2010) showing that scent marks of semi-free-ranging *Lemur catta* encode information about heterozygosity, and thus genetic quality as well as relatedness. *Propithecus verreauxi coquereli* and *L. catta* in the same facility have been studied for differences between the chemistry of the sexes (Hayes et al. 2004).

Thus, previous research has laid a good foundation for the context of scent-marking in prosimians and has begun to reveal the chemical basis of these marks in captivity, but has not yet elucidated the details for wild primates. Because environmental and social complexity can have a strong effect on odor signals, understanding the correlation between chemical communication and kin recognition in natural populations is important and it is not clear whether these signals could be communicated in the face of differences in diet and other complexities of the natural world. Therefore, we conducted an in-depth analysis of anogenital secretions collected from wild *Propithecus edwardsi* in Ranomafana National Park to identify specific volatile organic chemicals that singly or in groups might be characteristic of genetic relatedness and thus used in decisions regarding mate choice or nepotism.

Materials and methods

Study Species

The Milne-Edwards' sifaka, *Propithecus edwardsi*, is a large-bodied, arboreal lemur that lives in multi-male, multi-female territorial groups of 2-10 individuals and consumes a combination of leaves and fruit. The *P. edwardsi* inhabiting the subtropical rainforest enclosed by Ranomafana National Park (RNP) in southeastern Madagascar are among the most intensively studied animal populations in the world (Wright 1995; Wright 1999; Pochron et al. 2004; Irwin et al. 2005; King et al. 2005; Arrigo-Nelson 2006; Karpanty and Wright 2006; Dunham et al. 2008; Wright et al. 2008). *P. edwardsi* make ideal candidates for this research because of their obvious reliance on olfactory communication, scent-marking up to 30 times per day (Pochron et al. 2005a). Males and females have an anogenital scent gland and males have an additional pectoral gland.

Sample Collection

Scent samples were collected during the beginning of the breeding season (November 23-26, 2006) and again in the non-breeding/birthing season (June 7-13, 2007) from four habituated social groups of *P. edwardsi* living in the Talatakely Trail System of RNP as well as two groups living in a nearby, less disturbed area of forest (n = 14). We captured animals by darting with a CO_2 air rifle which injected Telazol® (a combination of the dissociative anaesthetic drug Tiletamine and the benzodiazepine anxiolytic drug Zolazepam) at 10 mg/kg body weight intramuscularly, employing a protocol used successfully since 1989

(Wright 1999). All animals were allowed to recover and released without incident after weighing, measuring, and collecting blood and other samples.

Excluding juveniles, we collected scent samples from eight females and six males (total scent samples = 28; Table 1). *P. edwardsi* were defined as adults (n = 8) if they were breeding in, or had dispersed from, their natal group; subadults (n = 6) were non-breeders that were older than 2 years and still in the natal group. To collect scent samples, we carefully swabbed the area of the scent gland of an anaesthetized lemur with 1 cm × 4 cm glass filter paper (after Salamon 1994; Zabaras 2003) using alcohol-swabbed metal forceps, two swabs per anogenital region per animal. The gland was not cleaned before swabbing, thus mimicking the natural state of glandular marking. The swabs were then sealed in an airtight vial and stored in a freezer at approximately -20 °C, (although there was at least one power outage during the months of storage) at the Centre ValBio research station adjacent to RNP. They were transported with a dry shipper to Stony Brook, NY, and stored again at -20 °C until they were finally shipped on dry ice to Hendrix College, Conway, AR. Samples were stored at -70 °C and thawed immediately before analysis by solid phase dynamic extraction (SPDE)/GC-MS (Lipinski 2001; Goodwin et al. 2007; Goodwin et al. 2012).

[INSERT TABLE 1 NEAR HERE]

Chemical Analysis

For SPDE/GC-MS analysis, each scent swab was sealed in a 20 mL screw-top vial with a threaded, metallic septum cap (silicone/PTFE layered septum;

www.autosamplerguys.com). Multiple samples were programmed to run automatically using the Combi PAL robot and associated SPDE hardware and software (www.chromtech.de). The SPDE needle was internally coated with activated charcoal (Carboxen®)-polydimethylsiloxane (AC-PDMS). After incubating the sample at 37 °C for 15 minutes, the headspace was extracted for 13 minutes (200 up-and-down one mL strokes of the gas-tight syringe). Desorption of analytes was at 250 °C in the GC inlet. GC/MS analyses were conducted using an Agilent 6890N GC and 5973N Mass Selective Detector. The capillary GC column was an Equity 1 (bonded; polydimethylsiloxane), 60 m × 0.32 mm ID, 1 μm film thickness (Supelco cat. No. 28058U). The GC oven was temperature programmed to hold for 2 min at 35 °C, followed by ramping to 180 °C at 3.75 °C/min where it was held for 5 min before ramping at 20 °C/min to a final temperature of 250 °C where it was held for 10 min. The mass spectrometer was programmed at 3.09 scans/sec for a mass scan of 30-500 amu. The identities of compounds reported herein were confirmed by comparison to the NIST02 mass spectral library.

Relatedness Analysis

Maternal relationships were known for most individuals from extensive pedigrees and were otherwise determined as per Morelli et al. (2009). In brief, we isolated genomic DNA from blood samples according to standard procedures (Sambrook et al. 1989) and conducted polymerase chain reaction (PCR) using 16 genus-specific microsatellite markers (from Lawler et al. 2001; Mayor et al. 2002; Rakotoarisoa et al. 2006) and unpublished markers developed by E. E. Louis and colleagues (see Morelli 2008). For pairwise estimates of relatedness, ML-

RELATE (Kalinowski et al. 2006) uses a maximum-likelihood approach to calculate the degree of relatedness between individuals, determining the likelihood of paternity for each male based on the genotypes of the focal individuals and the genotypes of individuals in the surrounding population, with output as a score based on the natural log of the overall likelihood ratio (Ln (Likelihood)). The results of these analyses are particularly robust because all individuals were genotyped from blood samples and all individuals and their corresponding samples were known, avoiding complications of pseudoreplication.

Statistical Analysis

The presence of peaks in the chromatograms and their relative areas were analyzed using nonparametric Bray-Curtis cluster analysis and multidimensional scaling (MDS) ordination (Clarke and Warwick 2001) to ascertain if animals clustered according to sex, season, social group or age class. Using relative percentage area for the peaks removes the need for standardizing concentrations from samples where slightly different total mass of components has been collected in a swab. Instead, it is the relative amount of each component that is compared thus ensuring that comparisons can be made between samples of unknown total concentrations. Each point in the MDS plot represents an individual swab, and points that are clumped correspond to individuals with similar peak composition (presence and abundance). Since they represent relative differences between samples, MDS plot axes are dimensionless. MDS has been used successfully in previous studies to analyze chromatographic data (e.g. Hayes et al. 2006; Nahrung et al. 2009). To determine whether clusters of individual lemurs were significantly different from each other, an analysis of similarity

(ANOSIM) was used. These tests depend only upon rank similarities, and thus are appropriate for these types of data. Software used for the multivariate analysis was Primer 5 for Windows (V 5.2.4, 2001).

Since we have previously reported significant differences between samples taken in different seasons (Hayes et al. 2006), we examined the similarity of repeat swabs from the same animal, within and between seasons (breeding and non-breeding), and compared to swabs from different animals. Significance was determined using Kruskal-Wallis and pairwise Mann-Whitney U tests, with significance determined at $P = 0.05$.

To determine whether there was an identifiable genetic signature in the scent mark, we compared a fourth-root transformed Bray-Curtis similarity matrix of the chemical data to the relatedness data. In the cases where animals were sampled in more than one period (June and November), we averaged the estimate for chemical similarity to get a single score of mean similarity. Using the full complement of swabs and then a subset of just related individuals ($r \geq 0.12$), we performed a correlation of relatedness (r) against chemical similarity. We then considered the samples collected in each season separately to control for inter-seasonal differences, and the samples collected at only one sampling location to control for inter-regional differences.

To identify the compounds contributing to relatedness-chemical similarity patterns, or compounds that may be heritable, for each individual we calculated the mean value for each compound. For every pair of related individuals, we determined the proportion of each compound found in each animal. These data

were transformed to provide a similarity score between 0 (completely dissimilar) and 0.5 (identical - because each individual contributed half of the total amount of that compound). A Pearson correlation between these values and relatedness was determined for each compound across all related individuals (n = 15 comparisons). Statistical analyses, unless otherwise specified, were performed using GenStat (V 11.1.0.1575, 2008).

Results

Results from multivariate statistical analyses revealed significant and characteristic clustering of compounds by sex (global R = 0.317, P = 0.004, Fig. 1a) and season (global R = 0.543, P = 0.001, Fig. 1b), but not with respect to social group (global R = 0.093, P = 0.16). Additionally, we identified a number of compounds that were common to the majority of samples and other compounds that appear only in samples from one sex, one season (breeding or non-breeding) or some combination of these (Table 2). These compounds may thus function as sexual or other signals, although this hypothesis awaits behavioral testing, and the small sample size in the present study precludes a rigorous statistical analysis of these trends.

[INSERT FIGURE 1 NEAR HERE]

[INSERT TABLE 2 NEAR HERE]

We found that repeated samples within a season from the same *P. edwardsi* were more similar than swabs from two different *P. edwardsi* from that same season (Table 3). This pattern held when the analysis was restricted to June samples

(Mann-Whitney U test: $U = 71.0$, $P < 0.001$). However, this similarity within an individual disappeared when swabs were compared across time; swabs from the same *P. edwardsi* in different seasons were no more similar than from two different *P. edwardsi* sampled in different seasons (Mean similarity score ± SEM) of repeated sampling of the same individual within season: 72.87 ± 1.13; of the same individual between seasons: 43.32 ± 1.01; of different animals within season: 40.11 ± 0.20; overall Kruskal-Wallis: $H = 23.36$, $P < 0.001$). Thus *P. edwardsi* appear to have an individually characteristic scent but it is superseded by a temporal signal.

The estimated value of r varied between 0 and 0.6 for pairs of focal animals, with an average of $r = 0.21$ (Table 3). Most sampled individuals were not related ($r < 0.12$). There was a significant positive correlation between chemical and genetic similarity for the swabs from the *P. edwardsi* ($n = 91$) ($r = 0.22$, $P = 0.034$), and this relationship was strengthened when we considered only related *P. edwardsi* ($n = 37$) ($r = 0.39$, $P = 0.017$; Fig. 2). To control for the significant inter-seasonal variation detected and any possible variation due to sampling location, we then considered only related animals from June (where we had a greater number of samples), and the samples from three adjoining social groups living in the Talatakely trail system (Talatakely III was not sampled during this time period). Again we demonstrated a positive relationship between relatedness and chemical similarity (June only: $r = 0.53$, $P = 0.042$; Talatakely only: $r = 0.48$, $P = 0.024$). Four compounds were significantly positively correlated with relatedness: 1-butanol, dimethyl sulfone, *N*-ethylaniline and unknown 10 (Pearson correlation $r = 0.54$, $P = 0.037$; $r = 0.60$, $P = 0.017$; $r = 0.62$ $P = 0.013$; $r = 0.54$, $P = 0.037$, respectively).

[INSERT TABLE 3 NEAR HERE]

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Discussion

Our results provide a clear demonstration of a correlation between chemical similarity and neutral genetic variation in a wild primate population. This result is remarkable given the potential confounding factors that could mask this signal in a natural setting, including differences in diet and behavioural complexity, as well as complications of field research including sampling and storage techniques and low sample size. Nevertheless, similar to studies in captive populations (Charpentier et al. 2008; Boulet et al. 2009; Charpentier et al. 2010), we found that semiochemical profiles were correlated with genetic distance.

We found strong temporal sex differences in scent chemistry as well, with many compounds found in only one time period and only one sex. In fact, these differences outweighed individual distinctions. Since individuals were sampled both in the breeding season (November) and the non-breeding season (June), this may indicate a breeding component to the scent mark. For example, dimethyl sulfone was detected in males and females only in the non-breeding season, and was also one of the compounds positively correlated to relatedness in our study. This compound is a known component of mammalian chemosignals, having been reported from chemical analyses of sheep wool (Burger et al. 2011), human skin (Gallagher et al. 2008) and rat urine (Osada et al. 2009). Seasonal aspects of food availability may also drive these differences.

Indeed, all of the compounds that we were able to identify that correlated with relatedness in this study have been reported from other mammalian chemosensory studies (e.g., *N*-ethylaniline in the feces of Iberian wolves (Martin et al. 2010) and the urine and vaginal mucus of white-tailed deer (Jemiolo et al. 1995)). As our samples were collected from the anogenital region, it is possible that this compound is derived from *P. edwardsi* feces, although still likely a component of the chemical message. Butanol is a component of rat urine (Osada et al. 2009) and human skin (Gallagher et al. 2008), and two species of primates have been shown to have an olfactory sensitivity to this alcohol (Laska and Seibt 2002). The urine of several species of strepsirrhine primates also contains butanol (Delbarco-Trillo et al. 2011).

We detected other components, including 1-octanol and phenol, in our study of the voided urine of these strepsirrhines. 1-Octanol was found only in males in the non-breeding season in our study and in the urine of 50% of *Eulemur macaco flavifrons* test subjects (Delbarco-Trillo et al. 2011). Phenol was found in every sample in both seasons in our study, and is also common to wolf feces (Martin et al. 2010), rat urine (Osada et al. 2009), human skin (Gallagher et al. 2008), rabbit chin gland secretion (Hayes et al. 2002) and the urine of the lemurs *L. catta* and *Eulemur coronatus* (Delbarco-Trillo et al. 2011).

Since collection and storage of samples in a remote field situation has obvious inherent difficulties, our study represents a conservative estimate of the correlation between relatedness and chemical similarity, scent distinctions between sexes, and other factors that we still found highly significant. The next

step will be to expand the data set, as well as to conduct behavioral bioassays with anogenital gland secretions and with specific chemical components thereof.

In terms of mate selection or nepotism, our results suggest that anogenital scent marks can encode information regarding relatedness between individuals. The research highlighted here gives a detailed view of how the chemical constituents of scent secretions could permit kin recognition and enable mate choice in a wild primate population. These results build on our earlier work where we showed that wild *P. edwardsi* males may scent mark to advertise to females (Pochron et al. 2005b). Understanding the information encoded in the scent mark is central to understanding Strepsirrhine communication, and can have important implications in managing wild population as well as those in captive breeding programs (Fisher et al. 2003). The ability of *P. edwardsi* to use these messages would allow them to assess genetic relatedness and make informed decisions regarding social interactions and mating strategies.

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Figure and Table Headings

Table 1: Source of scent samples; all animals were sampled in November 2006 and/or June 2007 in Ranomafana National Park, Madagascar.

Table 2: Compounds identified from swabs of *P. edwardsi* anogenital region. Retention time is given for each compound, as well as the number of swabs from which the compound was detected, separated by sex and season of the animal providing the swab. Superscripts identify compounds within several different categories.

Table 3: Double matrix showing genetic relatedness of *P. edwardsi* focal animals in the upper right portion, and mean chemical similarity between *P. edwardsi* swabs in the lower left portion. ID given for each animal is the same as used in Table 1.

Figure 1: Two-dimensional MDS ordinations of the 28 lemur swabs. The plot is based on fourth-root transformed abundances and a Bray-Curtis similarity matrix.

a: Swabs from the two sexes cluster separately (male - filled square, female - empty square)

b: Swabs from the two sampling seasons also cluster separately.(June - filled circle, November - empty circle)

Figure 2: Mean chemical similarity against relatedness for all related lemurs, there is a significant positive correlation ($r^2 = 0.38$, $P = 0.017$) between the axes.