Beyond Odor Discrimination: Demonstrating Individual Recognition by Scent in *Lemur Catta*

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Abstract

The current study demonstrates, for the first time, the occurrence of olfactory individual recognition in a nonhuman primate species. The empirical demonstration of recognition systems requires 1) a set of cues produced by the sender (expression component), 2) the perception of these cues by the receiver (perception component), and 3) a functional response by the receiver (action component). On the basis of this framework, we analyzed by gas chromatography 35 brachial secretions collected from 10 males of *Lemur catta*. Moreover, we performed habituation/discrimination tests to demonstrate the perception component, and we designed a specific bioassay, based on territorial competition, to highlight a functional response to individual odors. We demonstrated that recognition of conspecific odors goes beyond the perception of cues other than individuality (familiarity, kin, season, age, and rank) and that the receiver actually forms a mental representation of a specific individual by its scent.

Key words: bioassays, brachial gland, gas chromatography, individual recognition, *Lemur catta*

Introduction

Animal recognition can take place from the simplest level (species) through sex (male–female) and group membership (group mates–foreign) to the most specialized level such as recognition of social rank and individuals (Thom and Hurst, 2004). The ability to recognize individuals is important in mating systems to determine mate choice in order to avoid inbreeding and assess the possibility of reproductive success (Bradbury and Vehrencamp, 1998). Moreover, in species with complex social interactions the skill to identify individuals allows the recognition of long-term partners with consequences on the assurance of nepotism, on the establishment of dominance hierarchies and other competitive relationships, and on the maintenance of delayed reciprocal altruism (Trivers, 1971; Bradbury and Vehrencamp, 1998; Thom and Hurst, 2004).

In nonhuman primates, individual recognition has been demonstrated on the basis of visual and acoustic cues (cf. Cheney and Seyfarth, 1980, 1990; Parr et al., 2000); however, individual recognition by olfactory cues has not been definitively demonstrated yet. In primates, smell seems to be less important than other senses such as the visual and acoustic ones. But, even in primates, smell has not been abandoned (Laska et al., 2000). In fact, it is becoming increasingly clear from studies of both human and nonhuman primates that olfaction may, in fact, play a significant part in the regulation of a wide variety of primate behaviors (Epple, 1986; Laska et al., 2000). This is particularly true for strepsirrhines, which show several ancestral mammalian features such as a well-developed rhinarium, a large number of turbinates, a fully functional vomeronasal organ, and odor-producing skin glands (Schilling, 1979). Thus, the ability to recognize individuals by their scent could represent one of the fundamental mechanisms in regulating several aspects of strepsirrhine social life. In this paper, we searched for olfactory individual recognition in *Lemur catta*. Ring-tailed lemur males possess highly specialized brachial and antebrachial glands that are used to mark objects in the environment and to impregnate their own tails before waving it toward conspecifics. Moreover, both males and females apply genital secretions during anogenital marking (Jolly, 1966; Kappeler, 1998).

The empirical demonstration of individual recognition is a difficult matter; in fact, recognition systems require 1) a set of cues produced by the sender (expression component; Tsutsui, 2004), 2) the perception of these cues by the receiver (perception component; Mateo, 2004), and 3) a functional...
response by the receiver (action component; Liebert and Starks, 2004).

By gas chromatographic (GC) analyses, we verified the occurrence of the expression component in the highly specialized brachial secretion of *L. catta* in order to understand whether such secretion possessed unique chemical signatures. Scent marks carry both fixed and variable information about the owner (genomic and metabolic, respectively). We selected brachial secretions because specialized gland secretions are generally “hard wired” in the genome rather than expressed by metabolic and environmentally dependent factors (Bradbury and Vehrencamp, 1998; Hurst and Beynon, 2004). On the other hand, the fluctuating nature of metabolic cues (such as urine, feces, and general body odors) is likely to make a system based on such cues relatively unstable thus needing an incessant updating in recognition systems (Hurst and Beynon, 2004).

In primates, the perception component was demonstrated in some Callitrichidae and Lemuridae species (cf. Mertl, 1975; Harrington, 1976; Epple et al., 1976) by habituation/dishabituation tests. To verify the occurrence of the perception component, we employed habituation/discrimination tests (HDTs) (Johnston and Jernigan, 1994). Tests based on the habituation response have been widely used in the assessment of individual recognition (Johnston and Jernigan, 1994; Thom and Hurst, 2004; Mateo, 2006). However, this method may suffer from the limitation that a discriminatory response between two scents could indicate the perception of differences based on possible incidental cues (kinship, familiarity, rank, age, reproductive status, and rearing conditions) rather than on individuality. For this reason, we performed HDTs trying to eliminate as much as possible the confounding factors that might determine scent discrimination.

Even in this case, HDTs lack a functional context; in fact, the increase of investigation on a new stimulus only demonstrates the perception of the novelty of such stimulus without giving any information on odor interpretation by the subject (Thom and Hurst, 2004).

Low-inbreeding mate choice, pregnancy block, and Coolidge effect are the behavioral and physiological responses generally used to assess the action component (Thom and Hurst, 2004). However, these experiments have been mainly designed for rodents (Thom and Hurst, 2004) and are very difficult to carry out in primates. Furthermore, it is hard to design functional tests that are independent of incidental cues (kin, familiarity, and rank) as individual recognition requires previous experience between senders and receivers (Johnston and Jernigan, 1994; Thom and Hurst, 2004; Mateo, 2006). The housing rearing condition in which two *L. catta* groups were housed in the Pistoia zoo (Italy) permitted us to design a functional bioassay based on territorial competition (Hurst and Beynon, 2004) to check for the occurrence of the third component of the individual recognition system in *L. catta*.

## Methods

### Subjects and housing

The study was conducted from October 2004 to November 2005 in five captive groups of *L. catta*. The P1, P2, and P3 groups were housed in the Pistoia zoo (Tuscany, Italy); the F group in the Falconara zoo (Marche, Italy); and the L group in the Lignano zoo (Friuli, Italy). P1 and L were multimale/multifemale groups composed of 10 individuals (five males and five females) and 5 individuals (two males and three females), respectively; P2 and P3 were single male/single female groups; and F was an all-male group (eight males in 2004, two males in 2005). All the individuals under study were adults (older than 18 months) and were in good health. Reproduction was not regulated. The study groups were selected on the basis of the quality of housing conditions: all lived in facilities composed of outside grassy enclosures (P1 and P2 about 100 m²; P3 about 20 m², F about 70 m², and L about 600 m²) and indoor halls (P1 about 20 m², P2 about 10 m², P3 about 5 m²; F about 5 m², and L about 20 m²). In particular, the P1 and P2 groups utilized the same outside grassy enclosure in alternation from 4–6 h per day; the two groups were always in olfactory and visual contact near the doors that separated the indoor from the outdoor areas. Since group P3 and group P1/P2 were spaced apart, they were neither in visual nor in olfactory contact.

All the animals were able to move freely between indoor and outdoor areas; all the outside enclosures were equipped with trees, ropes, and platforms so that the animals could move in all three dimensions. Large glass windows in all the indoor enclosures allowed the lemurs to follow the natural day/night cycle, and during winter the indoor cages were heated by radiators. The animals received food (fruits, vegetables, yogurt, and monkey chow) twice a day.

### Collection of brachial gland secretions

We collected brachial secretions from one gland of two subordinate males of F group in May 2004. In May 2005, we collected secretions from both right and left brachial glands of seven adult males from the P1, P2, P3, and L groups and from the right gland of one adult male of the F group. Finally, in November 2005, we collected secretions from both right and left brachial glands of 10 males from the P1, P2, P3, L, and F groups. All the secretions were collected by squeezing the brachial glands. We prevented chemical contamination by directly collecting the secretion using a perfectly clean aluminum sheet thus avoiding any contact with hands. The samples were labeled with the name of the donor subject and the gland side (left/right). The samples were immediately frozen at −20°C until used. We used some of these secretions for scent trials (see below), whereas only the secretions collected from both right and left brachial glands were used for GC/flame ionization detector (FID).
Experiment 1—GC/FID analysis

About 1 µg of glandular secretion for each sample was suspended in 100 µl of extraction solvent (1:3 v/v methanol:chloromethane mix) for 2 min. We injected 2 µl of solution into a Varian 3900 gas chromatograph fitted with a FID and a fused silica capillary column coated with 5% diphenyl–95% dimethyl polysiloxane (Varian FactorFour VF-5ms 30 m × 0.25 mm × 0.5 µm). Injector temperature was 280°C and detector temperature was 300°C. The carrier gas was hydrogen (at 12 psi). The temperature protocol was 70–150°C at a rate of 30°C/min (held for 5 min) and 150–310°C at 5°C/min (held for 11.3 min).

Scent tests

Two pieces of filter paper of 3 × 5 cm² (with brachial secretion of about 1 mm³) were fixed to the gates of outdoor and indoor enclosures at a distance of 50 cm from each other using forceps. One of the two authors numbered the two pieces of paper. The second one performed a blind trial, presenting the filter paper to animals and registering olfactory responses without knowing the meaning of the two numbers. The observer waited until the animals spontaneously approached the samples. The experimental trials were considered valid only if the animal spent more than 10 s inspecting the two samples combined and if both pieces of filter paper were detected by the subject. Each trial lasted 3 min for each animal; when two animals simultaneously approached the stimuli, the first author timed the 3-min trial of the two different animals. During our experiments, it never occurred that more than two animals approached the stimuli simultaneously. Time spent investigating was tape recorded starting when the animal was about 2 cm from the scent stimuli and until the individual moved away. Since trials were performed on the whole group, we frequently changed the relative position (left/right) of the two samples during the trials so that previous experience and copying behaviors did not bias the scent tests. Moreover, if a subject countermarked one of the two samples, we changed both of them at the end of the trial performed by that subject. For all experimental trials, the names of individuals interacting and time spent in investigating (sniffing and/or licking) the two samples were recorded. Since the animals showed high variability in their motivation to investigate, we obtained different sample sizes for the different experiments performed. We performed one trial per animal.

Experiment 2—Scent discrimination

We performed the first experiment comparing the olfactory response elicited by the brachial secretion and clean paper to verify whether animals actually perceive the scents (experiment 2a).

To verify the occurrence of perception component, we performed HDTs as suggested by Johnston and Jernigan (1994) and Thom and Hurst (2004) (experiment 2b). During four habituation trials, subjects were presented with two pieces of filter paper both smeared with the same amount of brachial secretion from an individual (individual A). The habituation response is usually observed in the form of a decrease in the inspection time. In the final trial, we presented two different odors, belonging to the same individual (A) and to another individual (B). If the subject is able to perceive the difference between the new scent (B) and the habituated scent (A), investigation of the former is expected to be higher when compared to the latter (Thom and Hurst, 2004). The habituation trials were followed by 1-min intervals. We reversed the habituated and the new scent in the trials on different groups.

In order to reduce the possibility that animals could perceive differences due to extrinsic factors (age, rank, group provenance), we used the secretions from the two P1 subordinate males for the trials in F and L groups and the secretions from the two F subordinate males for trials in P1, P2, and P3 groups. As a consequence, the two individuals A and B were both unfamiliar to the test subjects. In addition, the two donors were age matched. Finally, each couple of secretions (A and B) was collected by the same procedures on the same day to avoid the bias due to possible seasonal differences (Hayes et al., 2005).

Experiment 3—Individual recognition by scent

To verify the occurrence of the functional component, we performed a bioassay based on two experiments on the two groups competing for the same outside enclosure (P1 and P2). In the first experiment, we presented a familiar scent belonging to a group mate and another one belonging to an unfamiliar subject from L group (experiment 3a). In the second experiment, we presented the familiar odor of the male belonging to the competing group and the unfamiliar male from P3 group (experiment 3b). Even though the male of group P3 comes from the same zoo of the groups P1 and P2, it is as unfamiliar as the male of group L. The competing and group-mate donors shared the same relatedness coefficient (0.5) with the experimental subjects. The scent tests were performed in the outdoor enclosure, which represents the overlapping area for P1 and P2 groups.

Statistical analysis

The peak areas of the FID gas chromatograms of each sample were processed and analyzed by Varian Star GC Workstation 6.0. Each peak was identified on the basis of the relative retention time in the 35 analyses; peak areas were transformed into percentages for each sample. All peaks with a percentage area less than 0.01% of the total compound content (considering all the samples) were excluded from the analyses because of unreliable quantification at such low relative amounts as suggested by Smith et al. (2001).
We performed discriminant analysis (DA) on 35 samples collected from 10 donors in different periods (mating and birth seasons) to determine whether the different samples from each animal could be distinguished according to their chemical composition. Using the procedures described in Sledge et al. (2001) and Sumana et al. (2005), we performed principal component analysis (PCA) to reduce the number of variables into smaller number of uncorrelated principal components. We extracted 16 factors with eigenvalues greater than 1, which together explained 100% of the total variance. As no peak showed communalities <0.8, we did not remove any peak from the PCA. The 16 principal components were used as independent variables for the DA. Wilks’ lambda and the number of cases assigned to their original group were used as indexes of correct DA.

To visualize the pattern of proximity among chemical profiles of the different individuals, we applied cluster analysis to the right gland secretions collected in November 2005 from 10 individuals. We used squared Euclidian distances as a dissimilarity measure (Z-scores were used to standardize the percentages), thus obtaining a dissimilarity matrix, which was subjected to a cluster analysis using unweighted pair group method with arithmetic mean method.

We used the Wilcoxon matched-pairs signed-ranks test to evaluate differences in time spent investigating during scent tests (blank paper vs. brachial secretion), HDTs (habituated vs. nonhabituated scent), and functional tests (familiar vs. unfamiliar scent). All analyses were two tailed, and the level of significance was set at 5%. We employed exact tests as suggested by Mundry and Fischer (1998).

**Results**

**Chemical analyses (experiment 1)**

A total of 108 peaks representing one or more chemicals reached the 0.01% in the GC/FID analyses of the glandular secretions of the 10 subjects. None of these peaks were found in the control analysis of the clean solvent. Among the 108 peaks, 99 were present in all the 10 individuals, 9 were absent in only 1 individual, 4 lacked in 2 individuals, and only 1 was absent in 4 subjects.

DA performed on the 35 samples using the 16 PCs obtained by PCA extracted eight functions explaining 100% of variance and correctly assigned 100% of cases to their original correct group. Particularly, on the basis of the function 1 (explained variance 54.5%, Wilks’ lambda = 0.000, \( P < 0.001 \)) and function 2 only (explained variance 20.1%, Wilks’ lambda = 0.000, \( P < 0.001 \)), it was possible to highlight a good separation of the samples according to the 10 individuals (Figure 1).

As 92% of the chemicals were shared among all the subjects, interindividual odor differences are mainly dependent upon the relative concentrations of the volatile compounds. A first identification of these compounds was performed by Hayes et al. (2005), which provided a list of compounds identified by mass spectrometry.

Cluster analysis separated two main clusters (Figure 2). Both clusters included individuals having different ranking position, different relatedness coefficient, and living in different groups characterized by diverse social situations (one male/one female; multimale/multifemale). This result suggests that rank, kinship, and group membership do not influence the chemical composition of brachial secretions (Figure 2).

**Scent discrimination (experiment 2)**

Lemurs investigated more frequently the filter paper containing brachial gland secretions compared to the clean paper (Exact Wilcoxon signed-ranks test: \( T = 0, \text{ties} = 0, N = 12, P < 0.001 \)) thus showing that they perceive the scent (experiment 2a).

After four habituation trials, lemurs decreased their investigation activity on the habituation scents (first vs. fourth trial, Exact Wilcoxon signed-ranks test: \( T = 0, \text{ties} = 0, N = 10, P = 0.002 \)), and in the last discrimination test they preferentially investigated the nonhabituated scent compared to the habituated one (Exact Wilcoxon signed-ranks test: \( T = 0, \text{ties} = 1, N = 10, P = 0.004 \)) (experiment 2b) (Figure 3).
Individual recognition by scent (experiment 3)

Lemurs belonging to P1 and P2 groups investigated preferentially unfamiliar brachial secretion compared to that belonging to a group mate (Exact Wilcoxon signed-ranks test: $T = 0$, ties = 0, $N = 8$, $P = 0.008$; three subjects of the group P1 did not respond, and the male of the group P2 was not tested since a group-mate male donor lacked) confirming previous studies (Ramsay and Giller, 1996; Palagi et al., 2005a). Conversely, subjects from both groups preferred to investigate the familiar odor belonging to the competing male (P1 male for P2 group, P2 male for P1 group) compared to the unfamiliar odor (Exact Wilcoxon signed-ranks test: $T = 3.5$, ties = 0, $N = 10$, $P = 0.012$; two subjects of the group P1 did not respond) (Figure 4).

Discussion

In primates, olfactory individual discrimination (the ability to distinguish between two scents) has been demonstrated, while olfactory individual recognition (the discrimination of one out of many known individuals by its scent) has been only supposed on the basis of chemical and behavioral data but never definitively demonstrated (cf. Mertl, 1975; Smith et al., 1997, 2001). In 1975, Mertl performed habituation/dishabituation tests on *L. catta* using antebrachial secretions showing that males distinguish between the scents from different individuals. However, Mertl (1975) did not provide any information on kinship, rank, and familiarity of the donors; thus, scent discrimination might be due to a combination of variables rather than chemical individuality (Thom and Hurst, 2004).

Our chemical analyses showed the existence of an individual profile for the scent mark of each male (expression component) thus providing a basis for *L. catta* individual recognition. This uniqueness is probably due to the differences in the relative concentration of the diverse compounds (the 10 subjects shared the 92% of the peaks) rather than in qualitative variations. Similar results were also found for common marmosets *Callithrix jacchus* (Smith et al., 2001). As predicted for scents encoding individual identity, the brachial signatures did not show any similarity pattern according to ranking status, age, or group provenance of the donors, thus suggesting that social and environmental situations do not affect the chemical composition of the brachial glands (Figure 2). The volatile components of male secretions were also individually unique throughout the seasons (Figure 1).

The discrimination between two scents of different subjects found by Mertl (1975) and by our HDTs (experiment 2) is probably based on the signal individual uniqueness rather
than on the integration of different information. A further important support for this hypothesis is that the animals distinguished between secretions belonging to two unfamiliar males having the same age, ranking position, and group provenance. Clearly, HDTs do not permit to exclude that other scent components, not detected by GC, are involved in scent discrimination (e.g., nonvolatile odorants like proteins and the major histocompatibility complex; see Belcher et al., 1990; Thom and Hurst, 2004).

Furthermore, HDTs are not sufficient to demonstrate individual recognition (Thom and Hurst, 2004). In fact, these tests only show that animals are able to discriminate familiar from unfamiliar scents not giving any information whether the receiver associates the perceived scent with its donor. In order to evaluate whether such association occurs in *L. catta*, we designed a bioassay based on the strong territorial competition of ring-tailed lemurs. In this species, the disruption of the troops due to the loss of the defended area often results in the death of several group members (Jolly and Pride, 1999), and consequently, males and females actively defend their own territories (Jolly, 1966). Olfactory behavior plays a fundamental role in territorial defense (Jolly, 1966): owners extensively mark their territories (mainly at boundaries) and spend a lot of time seeking and investigating conspecific deposits (Jolly, 1966; Mertl-Millhollen, 1986, 2006; Kappeler, 1998; Palagi et al., 2005a). Generally, an odor belonging to a novel unfamiliar individual (a potential competitor) elicits more intense olfactory responses compared to a scent belonging to a group mate (experiment 3a; Ramsay and Giller, 1996; Palagi et al., 2005a). However, in the two adjacent competing groups of the Pistoia zoo, the odor of the well-known competitor, despite its familiarity, elicited a stronger response compared to an unfamiliar donor. The result of the experiment 3b suggests the occurrence of a higher order processing system that categorizes stimuli according to their significance and not strictly by their sensory features (Johnston and Jernigan, 1994).

Although signature polymorphism is essential for the occurrence of recognition, the Crozier's paradox predicts that, in the absence of large benefits, selection may deplete the diversification of the cues, since individuals showing rare labels have a high probability to be rejected as foreign (Crozier, 1987; Elgar and Crozier, 1989). However, the capacity to recognize conspecific identity might bring considerable advantages both to the sender and the receiver especially if animals can remember and use information from previous encounters to moderate future responses. Gosling (1982) proposed that “the function of territory marking is to provide an olfactory association between the resident and the defended area which allow intruders to identify the resident when they meet and thus reduce the frequency of escalating agonistic encounters” (scent matching hypothesis). Within group members, stable linear dominance hierarchies are predicted to develop when individuals can remember the outcome of prior encounters (for an extensive review see Beacham, 2003). Moreover, individual recognition may play an important role for avoiding inbreeding and maintaining coalitions and reciprocal alliances (Trivers, 1971). *Lemur catta* lives in multimale/multifemale groups with a complex social structure characterized by a strict linear hierarchy (Jolly, 1966). In this species, there is some evidence that individual recognition may occur. For example, females absolutely avoid mating with their relatives (Pereira and Weiss, 1991). Moreover, now and then some group members repeatedly attack “a single target individual”; these unprovoked aggressions can last from a few days to several months, and they generally end with the forced eviction of the victim (Vick and Pereira, 1989; Palagi et al., 2005b). Furthermore, reconciliation (a form of affiliative interaction between former antagonists occurring shortly after an agonistic event; de Waal and van Roosmalen, 1979) has been recently described in ring-tailed lemurs (Palagi et al., 2005b). Probably, visual and acoustic cues other than the chemical ones are implied in *L. catta* individual recognition. However, this species is characterized by a complex olfactory system, and many intragroup social interactions are mediated by chemical communication (Jolly, 1966; Kappeler, 1998; Palagi et al., 2005a; Mertl-Millhollen, 2006). Thus, it seems plausible that pheromonal polymorphism, which gives each individual its unique olfactory signature, is strongly selected in ring-tailed lemurs. The capability to recognize the individual ownership, other than simply perceive the spatial and temporal pattern of scent deposits, may provide to visitor and resident lemurs continuous and fundamental information useful in making reproductive and competitive decisions (Gosling, 1982; Bradbury and Vehrencamp, 1988; Hurst and Beynon, 2004; Palagi et al., 2005a,b). In conclusion, although it is difficult to decide whether observations and/or experiments actually demonstrate the occurrence of a true individual recognition, the current study strongly suggests that *L. catta* olfactory investigation of the individual brachial secretions goes beyond the discrimination between familiar and unfamiliar odors. In fact, according to previous competitive experience, *L. catta* modulate the response to an odor on the basis of a mental representation of the individual producing such odor.

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