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Olfactory Communication, Mate Choice, And Reproduction In A Pair-Bonded Primate (aotus Spp.)

Andrea Marie Spence-Aizenberg
University of Pennsylvania, aspenc@sas.upenn.edu

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Abstract

Primates are typically considered microsmatic (i.e., having a relatively less developed sense of smell) when compared to other mammals, yet it is becoming increasingly clear that olfaction is an important sense involved in communication in numerous primate taxa, including humans. Still, compared to other social and mating systems, little is known about olfactory communication in strictly monogamous non-human primates. Here, a comprehensive approach using chemical, behavioral, and hormonal data is used to explore how putative olfactory signals may mediate the formation and maintenance of the social and sexual relationship between mates in a socially and genetically monogamous New World primate, the owl monkey (Aotus spp.). This dissertation couples data collected from a captive population of A. nancymaae, and from a wild population of A. azarae as part of the Owl Monkey Project, a long-term project in Formosa, Argentina. Chapter 2 includes a robust chemical analysis of volatile components in the glandular secretions of captive and wild owl monkeys, and identified sex, age, gland of origin, and possibly individual identity as biologically relevant information encoded in these secretions. Chapter 3 investigates potential chemosignals of relatedness. Captive owl monkeys differentially responded to odors based on the relatedness to scent-donor, suggesting a chemosignal of relatedness. Wild pairs showed greater estimates of genetic relatedness than expected with random mating, suggesting individuals in this population do not avoid inbreeding, and likely use some mechanism to recognize kin. Chapter 4 explores female fecundity as a potential chemosignal. Captive males discriminated between the reproductive phases of females using olfactory cues alone. However, behavioral and olfactory behaviors of both captive and wild breeding pairs showed these cues are of limited significance. Finally, chapter 5 takes a broader perspective, considering the role of sexual selection on olfactory communication in owl monkeys. Owl monkey olfactory traits are dimorphic, and this, coupled with the potential role chemosignals may play in reproduction and mate choice, suggest sexual selection has influenced chemical communication in owl monkeys. Still, the degree of dimorphism is reduced compared to other primates. This dissertation expands our knowledge of how olfactory communication may vary with social and mating patterns.

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OLFACTORY COMMUNICATION, MATE CHOICE, AND REPRODUCTION IN A PAIR-BONDED PRIMATE (*AOTUS* SPP.)

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Supervisor of Dissertation

_____

Theodore G. Schurr

Professor, Anthropology

Co-Supervisor of Dissertation

_____

Robert Seyfarth

Professor, Psychology

Graduate Group Chairperson

_____

Theodore G. Schurr, Professor, Anthropology

Dissertation Committee

Eduardo Fernandez-Duque, Professor, Anthropology, Yale University

Bruce A. Kimball, Member, Monell Chemical Senses Center
Research Chemist, USDA-APHIS-WS-National Wildlife Research Center

Theodore G. Schurr, Professor, Anthropology, University of Pennsylvania

Robert Seyfarth, Professor, Psychology, University of Pennsylvania

Claudia R. Valeggia, Professor of Anthropology, Yale University

OLFACTORY COMMUNICATION, MATE CHOICE, AND REPRODUCTION IN A PAIR-BONDED PRIMATE (*AOTUS* SPP.)

This thesis is dedicated to

my parents, who always prioritized my education

Dave, whose love and support kept me sane and motivated

Caleb, for giving me perspective

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ABSTRACT

OLFACTORY COMMUNICATION, MATE CHOICE, AND REPRODUCTION IN A PAIR-BONDED PRIMATE (*AOTUS* SPP.)

Andrea Spence-Aizenberg

Theodore Schurr

Primates are typically considered microsmatic (i.e., having a relatively less developed sense of smell) when compared to other mammals, yet it is becoming increasingly clear that olfaction is an important sense involved in communication in numerous primate taxa, including humans. Still, compared to other social and mating systems, little is known about olfactory communication in strictly monogamous non-human primates. Here, a comprehensive approach using chemical, behavioral, and hormonal data is used to explore how putative olfactory signals may mediate the formation and maintenance of the social and sexual relationship between mates in a socially and genetically monogamous New World primate, the owl monkey (*Aotus* spp.). This dissertation couples data collected from a captive population of *A. nancymaae*, and from a wild population of *A. azarae* as part of the Owl Monkey Project, a long-term project in Formosa, Argentina. Chapter 2 includes a robust chemical analysis of volatile components in the glandular secretions of captive and wild owl monkeys, and identified sex, age, gland of origin, and possibly individual identity as biologically relevant information encoded in these secretions. Chapter 3 investigates potential chemosignals of relatedness. Captive owl monkeys differentially responded to odors based on the relatedness to scent-donor, suggesting a chemosignal of relatedness. Wild pairs showed greater estimates of genetic

relatedness than expected with random mating, suggesting individuals in this population do not avoid inbreeding, and likely use some mechanism to recognize kin. Chapter 4 explores female fecundity as a potential chemosignal. Captive males discriminated between the reproductive phases of females using olfactory cues alone. However, behavioral and olfactory behaviors of both captive and wild breeding pairs showed these cues are of limited significance. Finally, chapter 5 takes a broader perspective, considering the role of sexual selection on olfactory communication in owl monkeys. Owl monkey olfactory traits are dimorphic, and this, coupled with the potential role chemosignals may play in reproduction and mate choice, suggest sexual selection has influenced chemical communication in owl monkeys. Still, the degree of dimorphism is reduced compared to other primates. This dissertation expands our knowledge of how olfactory communication may vary with social and mating patterns.

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CHAPTER 1: Introduction

Olfactory Communication and Anthropology

The most understudied form of communication by anthropologists, including primatologists, is olfaction (Classen, 1992; Heymann, 2006a). There are several reasons for the relative neglect of this mode of communication when compared to other modalities. First, there is a long-standing prejudice in philosophy and the sciences towards the sense of smell. It has been referred to as a "primitive" sense, and was considered less closely linked to intelligence and cognition than other senses (Guérer, 2002; Agapakis and Tolaas, 2012). Anthropological studies of olfaction have also been at risk of being dismissed as "frivolous and irrelevant" (Rasmussen, 1999, p 57). The lack of interest in this "primitive" sense was compounded by the numerous methodological challenges associated with studying olfactory behavior in humans. More specifically, in humans, the effects of olfactory cues can be difficult to assess particularly when these cues are often unconsciously perceived (Almagor, 1990).

In the many circumstances that do involve the conscious sense of smell, the evaluation of scent perception is confounded by extreme individual and cultural variation (Rasmussen, 1999; Candau, 2004; Ferdenzi et al., 2011). This flexibility in the perception of odorants among individuals, and cross-culturally, is of interest both from a cultural and neurobiological perspective. Although odorants themselves do not change their chemical structure, the percept, or mental impression of the odorant (Lundström and Olsson, 2010; Reed and Knaapila, 2010), can vary among individuals and across cultures. For example, while certain odorants, such as that of rotten food, tends to be universally disliked, many

other odorants may be perceived differently based on cultural norms and individual experiences (Classen, 1992). Furthermore, the processing of olfactory signals is closely linked to the limbic system, making the perception of odorants closely linked to memories and emotions, hence highly variable on an individual level (Almagor, 1990; Lledo et al., 2005; Hoover, 2010; Lundström and Olsson, 2010). From this perspective, the percept of the odorant, which would typically be termed the "odor," is both socially and individually constructed.

The potential role of olfaction in intra-species communication should arguably be of interest to anthropologists. Extensive research on human olfactory communication suggests humans may use chemosignals to influence mood, hormones, and possibly mate choice (Wysocki and Preti, 2004; Lübke and Pause, 2015). For example, the addition of body odor to a visual cue of emotion (a facial expression), alters the classification of the perceived emotion (Zhou and Chen, 2009). Human subjects are also able to correctly identify the scent of a person that experienced fear in a two-choice test (Ackerl et al., 2002). Exposure to the scent of another woman in an experimental setting alters the duration of menstrual cycles of subjects (Preti et al., 1986; Stern and McClintock, 1998). Men also respond differentially to the odor of women during the follicular phase, when women are most fecund, and odors from the luteal phase (Singh and Bronstad, 2001; Havlíček et al., 2006; Gildersleeve et al., 2012).

Body odor differs based on genetic relatedness, thus it has also been linked to kin recognition. Women can identify their sisters, based solely on body odor, at a greater than chance rate despite their lack of confidence in their ability to do so (Lundström et al., 2009). Perhaps most importantly, there is evidence that scents perceived by the receiver

convey meaningful information even if the receiver is unaware of these changes. For example, exposure to different types of human sweat differentially activates the amygdala, despite subjects reporting no conscious difference between the two scents (Mujica-Parodi et al., 2009).

Overall, the evidence suggests that, at some point in our evolutionary history, chemical communication between humans likely played an important role. Still, research on human chemical communication is limited. It is unclear if, and how, potential chemosignals may influence human behavior outside a laboratory setting. To better understand the evolution of chemosignals in humans, and across all primates, is necessary to document the diversity and similarities in traits across primates. Emery Thompson and Muller (2016: 16) argue that:

> [a]lthough both primate sexual behavior and its underlying neuroendocrine regulation are diverse, a number of specific behavioral and physiological features have predictably evolved in response to particular mating contexts. These features are valuable and reliable clues from which to infer the evolutionarily history of sexual behavior for a species.

It is possible that, similar to reproductive traits, chemosignals have "predictably" evolved with respect to mating context. To better understand the evolution of chemosignals within humans and other primates, we need a more comprehensive understanding of these signals across social and mating systems in primates.

My doctoral research explores the expression, detection, and function of putative olfactory signals and how they might mediate the formation and maintenance of the social and sexual relationship between mates in owl monkeys. In doing so, my work generates data that will expand our knowledge of olfactory communication in primates,

and ultimately provide greater context for understanding the evolution of olfactory signals in humans as well.

Olfactory Communication in Non-Human Primates

Primates, including humans, are typically considered microsmatic, i.e., they are considered as having a relatively less developed sense of smell. When compared to other mammals, primates exhibit a reduction in the features associated with the main and accessory olfactory system, including a larger proportion of non-functioning olfactory receptor genes (Rouquier et al., 2000; Young et al., 2002; Gilad et al., 2003a; b), and a smaller olfactory bulb relative to brain size (Stephan et al., 1988). These features have often been attributed to a decreased reliance on olfaction (Heymann, 2006a; Drea, 2015; Laska and Salazar, 2015), and heightened emphasis on visual cues. However, it has also been suggested that the morphological differences in olfactory traits do not directly translate into differences in olfactory ability among primates (Laska and Hudson, 1995; Smith and Bhatnagar, 2004). In fact, despite drastic differences in the number of functional olfactory receptor genes and in morphology, squirrel monkeys, macaques and humans are all able to perform equally well in discriminating between odors (Laska et al., 2005).

Certainly, the sense of smell plays a critical role in the daily lives of all primates. The ability to detect odorants, or chemical stimuli in the environment, can serve multiple purposes. The sense of smell is used to locate edible food (Bolen and Green, 1997; Bicca-Marques and Garber, 2004), and acts as a sentinel warning against dangers such as spoiled food (Reed and Knaapila, 2010).

The growing interest in olfactory signaling in non-human primates has demonstrated strong links between chemical communication, social behavior, and reproduction. It is becoming increasingly clear that olfaction is an important sense involved in communication in numerous primate taxa (Snowdon, 2004; Heymann, 2006a; Drea, 2015). For example, individuals possess a unique signature of body odors (Smith, 2006; Scordato et al., 2007; Setchell et al., 2010). The chemical composition of glandular secretions also encodes information related to sex in several primate genera (MacDonald et al., 2008; Setchell et al., 2010; Morelli et al., 2013; Greene and Drea, 2014; Vaglio et al., 2016). Along the same lines, the chemical composition of mandrill secretions is correlated to male rank and age (Setchell et al., 2010; Vaglio et al., 2016).

Odor also contains information related to an individual's genetic makeup. For instance, secretions chemically encode information regarding the major histocompatibility complex (MHC) in ring-tailed lemurs (Knapp et al., 2006). Mandrills also show greater similarities in odor profiles with similarities in the MHC and, to a lesser extent, pedigree relatedness (Setchell et al., 2011). Genetic relatedness and individual heterozygosity are also encoded in ring-tailed lemurs, although these associations are only apparent during the breeding season (Charpentier et al., 2008, 2010; Boulet et al., 2009a).

There is also substantial evidence that many non-human primates can signal reproductive status and fecundity through odors (Ziegler et al., 1987, 1993; Savage et al., 1988; Converse et al., 1995; Hayes et al., 2004; Scordato and Drea, 2007; Crawford et al., 2011; Greene and Drea, 2014). Research has shown that conspecifics can detect differences in these odors (Scordato and Drea, 2007; Charpentier et al., 2010; Crawford

et al., 2011), and that they may elicit behavioral or physiological changes in the odor recipient (Savage et al., 1988; Ziegler et al., 2009a). Still, it remains unclear whether these signals may be present in taxa that are strictly monogamous.

A Non-Human Primate Model for Chemical Communication: Owl Monkeys

Owl monkeys are a good model with which to expand our current understanding of chemical communication among primates. They possess an unusual suite of traits and behaviors that differ from other non-human primates for which chemical communication has been extensively studied. Accordingly, they represent a model that allows us to explore the role of olfactory signals in male-female relationships, providing an opportunity to transform our current understanding of olfactory communication within primates.

More specifically, adult male and female owl monkeys form close, long-term, social and sexual relationships that last many breeding seasons (Fernandez-Duque and Huck, 2013). Owl monkeys maintain an affiliative relationship and close proximity with each other, with few occurrences of aggression between them (Fernandez-Duque and Huck, 2013). All offspring in a wild population are sired by the resident adult male in each social group (Huck et al., 2014), suggesting extra-pair paternity is low or nonexistent. This social configuration differs greatly from that of mandrills, and ring-tailed lemurs, who are not monogamous, and even from the sometimes pair-living sifakas and callitrichids, who show much more flexible mating systems than owl monkeys.

Additionally, owl monkeys show minimal sexual dimorphism. There are no differences in body length, mass, or body color (Fernandez-Duque, 2011), and even the

external genitalia look remarkably similar. The only physical characteristics with an appreciable level of dimorphism are the canines – which can be up to 25% greater in length in males than females (Fernandez-Duque, 2011). The extent of dimorphism present in the sub-caudal glands is unknown. Although it has been reported that female subcaudal glands are less conspicuous than those of males (Hill et al., 1959), there are no data to support this view, and some females do display large and well-developed glands. Again, these features differ greatly from those of mandrills, who are extremely sexually dimorphic in body size and coloration (Setchell, 2016). They also differ from those of lemurs and sifakas, who exhibit clear sexual dimorphism related to olfactory physical traits (Schilling, 1979; Lewis, 2005; Pochron et al., 2005; Scordato and Drea, 2007) and olfactory behavior such as stink-fights (Jolly, 1966).

Olfactory behaviors, and the effect of chemical signals, have also been extensively studied in some callitrichids. Yet, the extreme dimorphism in chemical signals – with dominant females able to chemically suppress ovulation in subordinate females (Ziegler, 2013a) – is seemingly absent in owl monkeys (Corley et al., 2017). The degree of dimorphism present in owl monkey olfactory traits is unknown, and indeed is one of the goals of this study. Given the numerous ways in which owl monkeys differ from other primate models of chemical communication, the wild and captive populations of owl monkeys that I study offer good opportunities to explore the role that olfactory signals might have in mate choice, reproduction, and sexual selection in a monogamous taxon.

The potential of owl monkeys as a model for understanding how olfactory communication may influence the formation and maintenance of pair bonds is further

reinforced by the fact that owl monkeys show an array of characteristics indicating that chemical communication is an integral component of their behavior. Anatomically, they possess an olfactory bulb that is large relative to brain size, and like other platyrrhines, they have a vomeronasal organ (Hunter et al., 1984). Additionally, they have apocrine glands throughout the body (Hanson and Montagna, 1962), and a specialized subcaudal gland with hypertrophic sebaceous and apocrine glands that exhibit thicker and more densely planted stiff, specialized hairs (Hill et al., 1959; Hanson and Montagna, 1962). Chemically speaking, there is also evidence that information is encoded in subcaudal scent gland secretions. A preliminary study (i.e. small number of subjects and no controls) found that the chemical profiles from captive owl monkey scent gland secretions had unique signatures for individual identity, sex, and family membership (MacDonald et al., 2008).

Behaviorally speaking, patterns of scent-marking (rubbing scent glands on a substrate), partner-marking (rubbing scent glands on their pair mate), and inspecting (sniffing the anogenital region of their partner) are reported in captive and wild owl monkeys (Wolovich and Evans, 2007; MacDonald et al., 2008; Corley et al., 2014). When male owl monkeys are deprived of olfactory cues (by treating the nasal cavity), aggressive interactions with unfamiliar males greatly decrease (Hunter and Dixson, 1983). This observation suggests that the absence of an olfactory signal emitted by the unfamiliar male can no longer be detected and, therefore, does not stimulate aggressive behavior. Interestingly, immature individuals do not have well-developed subcaudal glands (Hill et al., 1959; Huck et al., 2011), and in the wild, juveniles engage in fewer olfactory behaviors than adults (Corley et al., in prep). Additionally, administration of

testosterone triggered the development of the subcaudal gland in a captive juvenile male (Dixson et al., 1980).

Together, these findings suggest that the subcaudal gland plays an active role in the behavior of adults, but not juveniles, and therefore most likely functions in a reproductive context. This view is corroborated by evidence that the location of scent marks within their home-ranges does not support the idea that scent marks function to defend territories or resources (Corley et al., in prep). Instead, scent marks are likely to be used primarily for inter-sexual communication within groups, or to potential mates.

Hypotheses

In this dissertation, I investigate the role of olfactory communication in the inter-sexual relationships of socially and genetically monogamous owl monkeys (*Aotus* spp.) (Huck et al., 2014). The morphological and behavioral evidence indicating that owl monkeys rely on olfaction for intra-specific communication is very strong, although it remains unknown what signals are produced and received. Using data on the behavior, endocrinology, and chemical signals of captive (*Aotus nancymaae*) and wild (*Aotus azarae*) owl monkeys, I explore multiple hypotheses that explain the mechanisms and functions of chemical communication in owl monkeys.

In Chapter 2, I explore the hypothesis that olfactory cues in owl monkey body odor are used to communicate with potential mates. Given the long-term relationships with seemingly infrequent opportunities for extra-pair paternity (Huck et al., 2014), cues of partner quality, such as sex, age, or relatedness, are expected to be particularly important. Specifically, I test the predictions that captive *A. nancymaae* and wild *A.*

azarae individuals can be discriminated by sex and age based on the chemical content of their glandular secretions, and that cues of relatedness and individual identity will be evident in their chemical profiles. In addition to testing predictions derived directly from this hypothesis, I also use this data set to explore other characteristics potentially signaled in odor, including housing location and contraception status in a captive population. The large number of samples from both populations offers an opportunity to directly compare and contrast similarities and differences in the potential chemosignals present in these populations.

In Chapter 3, I extend the hypothesis proposed in Chapter 2. Specifically, I hypothesize that owl monkeys use chemosignals to recognize kin, and subsequently employ them in mate choice. We have observed that males and females encounter close relatives in the groups they try to join following natal dispersal (Fernandez-Duque, 2009). In fact, one female left her group once her brother became the resident male (Fernandez-Duque, 2009), suggesting that owl monkeys avoid mating with close kin (defined here as parent, offspring, or full sibling). In this case, it is reasonable to presume that signals of relatedness may be used in partner selection, as is observed in socially monogamous beavers (Sun and Müller-Schwarze, 1997). In fact, the actual process of mate choice in owl monkeys remains a mystery. Individuals who die or are evicted from their territory are replaced very quickly (Fernandez-Duque, personal communication), and for this reason understanding the process that leads to an individual replacing the former resident are largely unknown.

In this chapter I use two different approaches to evaluate this hypothesis. First, to test whether individuals can discriminate between the odors of individuals based on

estimates of relatedness, I conducted behavioral bioassays with individuals in a captive *A. nancymaae* population. The ability to discriminate between close kin and non-kin would suggest there is a chemical signal for relatedness. Next, I examined whether owl monkeys show evidence of inbreeding avoidance or preference. Either outcome would suggest that owl monkeys can discriminate kin from non-kin, and prefer to, or avoid, mating with close kin. Long-term monitoring of the wild *A. azarae* population, and previous work done developing microsatellites in this population (Babb et al., 2011) and establishing parent-offspring relationships (Huck et al., 2014) made this assessment feasible. Together, these approaches allow me to conduct a more comprehensive analysis of whether owl monkeys can discriminate kin using chemical cues, and whether kinship influences mate choice in the wild population.

In Chapter 4, I investigate the hypothesis that *Aotus* females produce a chemosignal of fecundity, and that this signal is perceived by males. Historically, it has been proposed that monogamy and pair bonding co-evolved with concealed ovulation, or the lack of fecundity cues (Morris, 1967; Alexander and Noonan, 1979; Lovejoy, 1981). An alternative possibility is that a signal of ovulation to a male partner would increase the probability of conception by focusing his sexual behavior on a time when conception is most likely to occur. Signals of fecundity could also be advantageous for males if they increase paternity certainty by concentrating mate-guarding efforts to the time when a female is most fecund. Evidence from pair bonded non-human primates, such as gibbons (Barelli et al., 2007) and callitrichines (Converse et al., 1995; Ziegler et al., 2005), has shown that females produce cues (visual and olfactory respectively) associated with ovulation. The existing evidence from owl monkeys seems to point to a system of

precisely timed copulations. Only eight instances of matings were observed in over 2,000 hours of observations of five wild pairs (Fernandez-Duque et al., 2002), yet offspring are regularly conceived during the breeding season in established pairs (Fernandez-Duque and Huck, 2013). I propose that olfactory communication plays an important role for owl monkey mates, as has been suggested for callitrichines (Snowdon et al., 2006), and that females signal fecundity to males via olfactory cues. To evaluate this hypothesis, I use behavioral data collected from breeding pairs of captive and wild owl monkeys while the females were simultaneously being monitored hormonally to estimate the timing of fecundity. Additionally, I use behavioral bioassays to evaluate if males respond differentially to odors from females based on their ovulatory phase. If they do, then this would suggest that males can detect female fecundity.

Finally, in Chapter 5, I hypothesize that chemical communication, an integral component of inter-sexual communication in *Aotus*, has been influenced by sexual selection. Given the monogamous social and mating pattern of owl monkeys, the lack of dimorphism in most visual characteristics, and similar levels in the intensity and frequency of intra-sexual competition during resident male or female replacements (Fernandez-Duque and Huck, 2013), it would not necessarily be expected that directional selection in relation to sex would have occurred in traits involved in olfactory communication. However, preliminary evidence indicates there is dimorphism in the chemical structure of odor (MacDonald et al., 2008). Thus, if body odor is used in mate choice (as proposed in Chapters 2 and 3) or to signal fecundity (as suggested in Chapter 4), then the production of olfactory signals may have undergone directional sexual selection. As a result, males and females may exhibit dimorphism in traits related to the

expression of chemical signals (gland anatomy, chemical profiles, depositing or inspecting behaviors). Such evidence of dimorphism in traits related to olfactory communication would suggest that there are differential selection pressures, via inter- or intra-sexual selection, on males and females of these species.

In this project, I have begun to examine this hypothesis with a qualitative and quantitative examination of physical and behavioral olfactory traits in captive Nancy Ma's owl monkeys (*A. nancymaae*). In examining the level of dimorphism present in traits related to chemosignaling, I will directly assess two of Snowdon's (2004) five criteria for identifying a sexually selected trait: the sexually selected trait is dimorphic and there is intra-sexual variation of the trait. I will assess the degree of dimorphism present in the appearance of the subcaudal and perianal regions and in olfactory behaviors. This provides a foundation to continue to evaluate the potential role sexual selection may have played on chemosignals in owl monkeys.

This thesis provides an extensive set of novel information and analyses, which will improve our understanding of olfactory communication, behavior, and biology of owl monkeys. This is the first extensive chemical analysis of glandular secretions from captive and wild platyrrhines. The chemical analysis, coupled with the two-choice behavioral bioassays – the first conducted in owl monkeys – will begin to identify putative chemosignals used by owl monkeys. This is the first investigation of relatedness between males and females in wild pairs, which can inform us how relatedness may influence mate choice and pair formation. Finally, this is the first assessment of sexual dimorphism in traits related to olfactory communication in owl monkeys. Ultimately, the combination of data from captive and wild owl monkey taxa will also contribute to a

comprehensive and improved understanding of chemical communication in owl monkeys, and across primates in general.

CHAPTER 2: Chemical composition of glandular secretions from a pair-living monogamous primate: Sex, age, and gland differences in captive and wild owl monkeys (*Aotus* spp.)

Abstract

Broadening our knowledge of olfactory communication in strictly monogamous systems can inform our understanding of how chemosignals may facilitate social and reproductive behavior between the sexes. Compared to other social and mating systems, relatively little is known about olfactory communication in strictly monogamous non-human primates. Furthermore, platyrrhines are not well represented in chemical analyses of glandular secretions. We conducted semi-quantitative headspace gas chromatography with mass spectrometry to investigate the chemical components of glandular secretions from the subcaudal and pectoral glands of a strictly pair-living platyrrhine, the owl monkey (*Aotus* spp.). In this study, the first chemical analysis of a wild platyrrhine population, our goals were to 1) conduct a robust analysis of glandular secretions from both captive and wild owl monkey populations, 2) identify whether biologically relevant traits are present in glandular secretions, and 3) compare and contrast the results between two *Aotus* species in different environmental contexts: wild *Aotus azarae* (N=33) and captive *A. nancymaae* (N=104). Our findings indicate that secretions from both populations encode sex, gland of origin, and possibly individual identity. These consistent patterns across species and contexts suggest that secretions may function as chemosignals. Our data also show that wild *A. azarae* individuals are chemically discriminated by age (adult or subadult). Among the captive *A. nanycmaae*, we found

chemical differences associated with location, possibly caused by dietary differences. However, there was no noticeable effect of contraception on the chemical profiles of females, nor evidence that closely related individuals exhibit more similar chemical profiles in *A. nancymaae*. Overall, our chemical differences associated with location, were possibly caused by dietary differences. However, there was no noticeable effect of contraception on the chemical profiles of females, nor evidence that closely related individuals exhibit more similar chemical profiles in *A. nancymaae*. Overall, our data suggest that glandular secretions of both wild and captive *Aotus* spp. convey specific information. Future studies should use behavioral bioassays to evaluate the ability of owl monkeys to detect signals, and consider whether odor may ultimately facilitate social and sexual relationships between male and female owl monkeys.

Introduction

Evidence of the critical role that chemosignals play in primate social behavior has been steadily increasing since the 1970s. In the past decade, research on non-human primate olfactory communication has flourished, dispelling the notion of the "microsmatic" primate (Heymann, 2006a; Laska and Salazar, 2015). Despite having smaller olfactory bulbs relative to brain size (Stephan et al., 1988) and a larger proportion of non-functioning olfactory receptor genes (Rouquier et al., 2000; Young et al., 2002; Gilad et al., 2003a; b) compared to other mammals, these morphological differences in primates do not directly translate to differences in olfactory ability (Laska and Hudson, 1995; Smith and Bhatnagar, 2004). In fact, chemical evidence from non-human primate taxa suggest there are individual signatures of body odors secreted from scent glands, and that these odors encode information related to sex, age, rank, reproductive status, and genetic makeup (Drea, 2015). There is also substantial evidence that conspecifics can detect differences in these odors, and such odors may elicit behavioral or physiological changes in the odor recipient (Drea, 2015). More importantly, odor has been linked to variables (i.e. rank) important for mate choice in mandrills (Setchell, 2016). Odors are used in direct intra-sexual competition through stink-fights in ring-tailed lemurs (Jolly, 1966) and reproductive suppression in some callitrichines (Ziegler, 2013b). As a first step to identify potential chemosignals in a strictly socially monogamous pair-living platyrrhine, we investigate the chemical components of glandular secretions in owl monkeys (*Aotus* spp.).

It seems likely that olfactory communication plays an integral role in intra-specific communication in owl monkeys that, like other platyrrhines, have scent glands (Hill et

al., 1959; Hanson and Montagna, 1962) and vomeronasal organs (Hunter et al., 1984). Yet, among platyrrhines extensive research has been limited primarily to callitrichines (Heymann, 2006b). And while studies of callitrichines indicate that chemosignals affect both behavior and physiology of individuals by increasing sexual behavior based on fecundity cues in odor (Ziegler et al., 1993; Converse et al., 1995), suppressing ovulation of subordinate females (Epple and Katz, 1984; Savage et al., 1988; Barrett et al., 1990), or modifying testosterone production in males (Ziegler et al., 2011), evidence of chemosignals are not yet available for most platyrrhine taxa. Moreover, only two published studies, in common marmosets (N=5 individuals, Smith, 2006) and owl monkeys (N=13 individuals, MacDonald et al., 2008), have investigated the chemical composition of glandular secretions in platyrrhines, and there have been no such studies of wild populations. This project is the first to chemically evaluate glandular secretions in platyrrhines with such a robust sample size, and the first to include a wild population.

The study also offers an opportunity to evaluate the glandular secretions of pair-living monogamous primates. To better understand the mechanisms and function of chemosignals in the context of mate choice throughout the primate clade, it is necessary to explore the function of putative chemosignals in different social and mating systems. To date, most studies have focused primarily on non-monogamous taxa, such as lemurs or mandrills, and cooperative breeders, such as callitrichines, all of which display different social and sexual relationships than those observed in owl monkeys. Owl monkeys are strictly socially monogamous, establishing multi-year relationships with no evidence of extra-pair reproduction (Huck et al., 2014). Given these differences in social and mating systems, it is reasonable to expect that chemosignals may function differently

in owl monkeys than in non-monogamous taxa or species with more flexible mating relationships. When individuals form multi-year relationships, as in *Aotus*, an individual's reproductive success will be highly dependent on their breeding partner for several breeding seasons. In this case, we might expect that cues of individual quality are equally, or even more important, in pair-living taxa than in those for which the reproductive success of an animal is associated with mating with multiple partners. It is also possible that odor from glandular secretions are not primarily used to signal quality or traits used in mate choice, or to directly compete with conspecifics, but to facilitate the long-term bond between pair mates. Olfaction is an essential component of bonding in pair-living socially monogamous prairie voles (*Microtus ochrogaster*), where the removal of the vomeronasal organ or the olfactory bulb diminishes the development of partner preference between individuals (Williams et al., 1992; Curtis et al., 2001). Identifying how chemosignals function in pair-living, socially monogamous taxa can help elucidate whether olfactory communication, and the associated physical traits, operate similarly across primate social and mating systems, or instead, whether they represent derived traits.

In this study, our goals were to 1) conduct a robust semi-quantitative chemical analysis of glandular secretions in a platyrrhine genus (*Aotus*), including the first analysis of samples from a wild platyrrhine population, 2) identify individual characteristics that may be encoded in the glandular secretions of *Aotus*, and 3) compare and contrast how these putative chemosignals differ between two species of *Aotus* in a captive (*Aotus nancymaae*) and wild (*A. azarae*) context. Owl monkeys (*Aotus* spp.) represent a good model species to investigate the potential role of olfactory communication in regulating

male-female relationships and pair bonding. Anatomical and behavioral evidence strongly suggest olfactory communication is important for them. Anatomically, they possess both an olfactory bulb that is large relative to brain size and a vomeronasal organ (Hunter et al., 1984). They also have apocrine glands throughout the body (Hanson and Montagna, 1962), and a specialized subcaudal gland (Figure 1) with hypertrophic sebaceous and apocrine glands that exhibit thicker and more densely planted stiff, specialized hairs (Hill et al., 1959; Hanson and Montagna, 1962). Behaviorally, both captive and wild individuals regularly display patterns of scent-marking (rubbing scent glands on a substrate), partner-marking (rubbing scent glands on their pair mate), and inspecting (sniffing the anogenital/subcaudal region of their partner) (Wolovich and Evans, 2007). Experimental manipulations have shown that when male owl monkeys are deprived of olfactory cues, aggressive interactions with unfamiliar males decrease (Hunter and Dixson, 1983). Finally, owl monkeys' glandular secretions are chemically rich, and it has been suggested by a study of a small number of individuals (N=13) that they may contain information related to sex, age, and family group (MacDonald et al., 2008).

When considering our second goal of identifying information encoded in secretions, we hypothesized that olfactory cues in owl monkey body odor are used to communicate with potential mates. Specifically, we propose that these odors signal information that would be useful when choosing a partner. Under this hypothesis, we predicted that the odor of individuals would be statistically discriminated by sex and age category – as seen in a preliminary study of *Aotus* (MacDonald et al., 2008), lemurs (Scordato et al., 2007; Morelli et al., 2013; Greene and Drea, 2014), and mandrills (Setchell et al., 2010; Vaglio

et al., 2016). Signals of relatedness may also be useful given the duration of owl monkey breeding relationships, the relatively infrequent opportunities for extra-pair mating, and the natal dispersal of males and females (Fernandez-Duque, 2009). Therefore, we predicted that close-kin dyads would have more similar chemical profiles than non-kin dyads, if inbreeding avoidance is mediated by olfactory cues, as is the case with socially monogamous beavers (Sun and Müller-Schwarze, 1998). Finally, if odors were individually identifiable, we would expect these signals to be somewhat stable over time and gland type, and predicted that there would be less intra-individual than inter-individual variation in chemical profile.

In addition to testing these four predictions, we also evaluated other variables not directly related to our hypothesis that may influence odor. First, given the differences in the frequency of scent-marking between the glands (Corley et al., in prep; Spence-Aizenberg et al., submitted; Wolovich and Evans, 2007), the appearance of the glandular secretions from these glands (Spence-Aizenberg et al., unpublished data) and the chemical differences of gland type found in ring-tailed lemurs (Scordato and Drea, 2007), we evaluated whether secretions originating from the subcaudal and pectoral gland could be discriminated statistically. Additionally, we examined whether individuals could be statistically discriminated by location within the colony given some differences between colony rooms in the ambient environment or diet. We also tested for effects of contraception, which has been shown to alter the chemistry of secretions in lemurs (Crawford et al., 2011).

Finally, by evaluating putative chemosignals in two different species and contexts, we have the ability to evaluate whether there are similarities or differences across these taxa

and environments. The multi-year monitoring of wild (Owl Monkey Project, Argentina) and captive owl monkey populations (Owl Monkey Breeding and Research Resource, DuMond Conservancy) allow us to complement the intensive sampling and experimental approaches possible in captivity with ecological studies of wild individuals to better understand the adaptive value of putative chemosignals. A combined field-lab approach has already proved valuable in understanding food sharing (Wolovich et al., 2006; Wolovich and Perea-Rodriguez, 2007), mortality trajectories (Larson et al., 2016) and circadian biology (Fernandez-Duque et al., 2010; Fernandez-Duque, 2012). Similar patterns in these two populations would allow for more robust interpretations of the results than a study of only one species or environmental context.

Methods

Study Sites and Subjects

We studied *Aotus nancymaae* (N=104) housed at the Owl Monkey Breeding and Research Resource (OMBRR) located in the Keeling Center for Comparative Medicine and Research (MD Anderson Cancer Center, University of Texas, Bastrop). The OMBRR houses approximately 400 owl monkeys on a semi-reversed light cycle with periods of darkness extending approximately from 1500h to 0000h. Animals are housed in one of two large colony rooms (North and South room), or a third smaller room. Animals are housed in pairs or family groups in enclosures approximately 1.8m^3 in size, while a few individuals are housed alone. Water is always available to the animals, and they are fed LabDiet® Fiber-Plus® Monkey Diet 5049 (LabDiet; St. Louis, MO) with fruit or vegetable twice daily before 1500h, which remains available throughout the dark cycle.

While enclosures are directly adjacent to one another, groups are isolated visually from each other, and white noise (a waterfall) buffers the acoustic interactions within the rooms. Groups may be within olfactory range of their neighbors, but only in direct contact with their cagemates. Some adult females were administered monthly intra-muscular injections of a hormonal contraception (N=16), medroxyprogesterone acetate (MPA). Because there were no marked differences in the gland secretion chemistry between non-contracepted and contracepted females (see below), samples from all females were included in the analyses.

We also studied a population of *Aotus azarae* (N=33) ranging in gallery forests along the Pilagá and Guaycolec rivers in Formosa, Argentina (58° 11′W, 25° 58′S). This population has been monitored regularly since 1997 as part of the Owl Monkey Project. The low levels of sexual dimorphism in *Aotus* (Fernandez-Duque, 2011) make it necessary to mark individuals to reliably and regularly identify them. To do this, animals in this population are darted and anesthetized using ketamine hydrochloride projected from a CO_2-powered rifle and fitted with VHF radiocollars, or ball-chain collars with colored beads, to facilitate individual identification, following established methods (Fernandez-Duque and Rotundo, 2003; Juarez et al., 2011).

This research on the captive *A. nancymaae* was approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee (ACUF# 05-13-04881). The Owl Monkey Project has had continued approval for all research on *A. azarae* presented here by the Formosa Province Council of Veterinarian Doctors, the Directorate of Wildlife, the Subsecretary of Ecology and Natural Resources and the Ministry of Production. At the national level, the procedures were approved by the National Wildlife

Directorate in Argentina and by the IACUC committees of the Zoological Society of San Diego (2000–2005) and of the University of Pennsylvania (2006–2013). All research adhered to the legal requirements of the United States of America.

Data Collection

One of us (ASA) collected 296 glandular secretions from 52 male and 52 female *A. nancymaae* during June – August 2013 (Table 1). Subjects ranged in age between 27 months and 25 years, and were defined as adults (>48 mos.) or subadults (24.1-48 mos.; Huck et al., 2011). The birthdates of two captive adults were unknown. We collected secretion samples from manually restrained animals by rubbing a sterile cotton swab over their subcaudal and/or pectoral scent gland back and forth five times following MacDonald et al. (2008). After collection, we sealed the swabs in a glass chromatography vial and stored them at -20°C (MacDonald et al., 2008; Drea et al., 2013). We collected a control swab (a swab exposed to the air) daily in each colony room where we sampled the animals. We shipped all samples on dry ice from the OMBRR to the University of Pennsylvania Reproductive Ecology Lab (Penn REL), where they were stored until analysis at the Monell Chemical Senses Center (Monell).

We also collected glandular secretions from 16 male and 17 female *A. azarae* wild individuals (but see also Appendix 2), with ages estimated between 16 months to 14 years, although seven adults were of unknown age. Their ages were defined as adults (>48 mos.), subadults (24.1-48 mos.) or juveniles (6.1-24mos.; Huck et al., 2011). Of the 72 samples collected from 33 individuals, we collected five (7%) of them between 2001-2007, and the remaining 67 (93%) between 2010-2013. We collected the scent gland

samples while individuals were anesthetized for a physical exam conducted following their capture (Fernandez-Duque and Rotundo, 2003; Juarez et al., 2011). Because captures require darting and anesthetization, we try to limit the number of individuals captured. Therefore, collection of glandular secretions are opportunistic and individuals may not contribute equally to the total sample. During physical exams, we rubbed sterile cotton swabs on the subcaudal and/or pectoral glands, stored them in separate glass vials, and transferred them to an off-site freezer within a few hours. We transported the samples at ambient temperature to the United States, then stored them at -20°C in the Penn REL until they were analyzed at Monell. We transferred the swabs to chromatography vials at Monell immediately prior to analysis.

Data Analyses

Headspace Analysis and Identification

We conducted all odor analyses in Dr. B. Kimball's lab at Monell. We considered the *A. nancymaae* and *A. azarae* samples separately in both chromatographic and statistical analyses. To characterize the volatile components of collected secretions, we subjected the swabs to dynamic headspace analysis combined with gas chromatography-mass spectrometry (GC-MS). Headspace analyses were conducted with an HT3 dynamic headspace analyzer (Teledyne Tekmar, Mason, OH, USA) using a Supelco Trap C desorption trap attached to a Thermo Trace GC-MS with a single quadrapole mass spectrometer and a 30 m 0.25 mm id Stabiliwax-DA fused-silica capillary column (RESTEK). Samples were maintained at 40°C, and swept with helium for 30 min at a 75ml/min flow rate. Volatiles collected on the trap, which were desorbed at 180°C. The

GC oven had an initial temperature of 40°C which was held for three min, then increased 7°C per minute to a final temperature of 230°C, which was held for 5.86 minutes. The MS was used in scan mode from 33-400 m/z. We used Xcalibur to convert the chromatographic data to NetCDF files, and Metalign (Lommen, 2009) for baseline correction, noise reduction, and peak alignment. We used MSClust (Tikunov et al., 2012) to identify peaks, and to generate a chromatographic response based on chromatographic peak height. Empty vials and control samples were used to detect for contaminants (Drea et al., 2013). We excluded from further analyses peaks with the largest peak heights in empty vials and control samples, as they were likely derived from the cotton swabs, chromatography vials, or the thermal desorption trap. Additionally, we removed peaks detected in less than 10% of samples and duplicate peaks (representing the same compound). Peaks IDs are based on their scan number in the chromatogram (Table 2).

We calculated the relative abundance for the remaining peaks in ≥10% of samples (N=110 peaks) based on the sum of these peaks (referred to here as the total chromatogram area), allowing us to control for any variation in absolute abundance that might be due to the amount of secretion collected. We used these peak values to estimate chemical distances, with the values being square root transformed, centered, and scaled for all classification analyses to reduce the number of uni-variate outliers for all classification analyses. For peaks included in models, we confirmed identifies of eight peaks using authentic standards (Table 2, also see Supplementary Materials) and relied on tentative identifications provided by the NIST Standard Reference Database 1A (US Department of Commerce, Gaithersburg, MD, USA) for all peaks we were not able to identify with standards.

Using principal component analysis, we identified outliers beyond the 95% confidence interval when plotting samples according to sample type using the first two components ("prcomp" function in R "stats" package, "ggord" in the package "ggplot2" in R). Identification and removal of outliers is critical when using linear discriminant analysis (LDA) because it is highly influenced by them. We had four samples in the captive data set (N=2 females, 2 males), and four samples in the wild data set (N=3 males, 1 female) whose values fell beyond the 95% confidence interval, and excluded these samples from statistical analyses. We conducted statistical analyses in R version 3.2.1 R (R Development Core Team, 2016).

Classification of Chemical Data

To test whether glandular secretions encode information of age category, sex, gland type, and housing, we used these four variables as dependent variables in linear discriminant analysis (LDA), to assess how well the chemical content of gland secretions can accurately classify samples into the pre-existing categories (dependent variables) (Drea et al., 2013). Based on our predictions, we expected to statistically discriminate individuals in both populations based on sex and age. When testing the classification of sex and age categories (adult: >48 mos. or subadult: 24.1-48 mos.; Huck et al., 2011), we used only subcaudal samples in the captive populations, but pooled the subcaudal and pectoral samples in the wild population because of the relatively small number of sampled individuals. We limited the analysis of gland type (subcaudal or pectoral) to adult and subadults, excluding the wild *A. azarae* juveniles because the number of subjects were so few. Location within the captive colony (North or South room) was used

as a dependent variable in the LDA to evaluate signals of housing, and the samples were limited to the subcaudal secretions of individuals only housed in these two rooms. Additionally, to minimize the potential confounding factors of the predicted chemosignals of housing, age, and sex, we balanced, as much as possible, the number of individuals of each age and sex sampled in each room (North room: 30 adults,13 subadults, 22 males, 21 females; South room: 34 adults, 8 subadults 18 males, 24 females).

To conduct the LDAs, we first controlled for pseudo-replication of samples in the cases where multiple samples of the same gland had been collected from the same individual, to avoid increasing the risk of a Type 1 error (Setchell et al., 2010). After finding no ability to discriminate samples based on the month in which it was collected among the *A. nancymaae* (samples could not be accurately sorted in a LDA based on collection month, with a correctness rate of only 52% using five peaks), we computed averages of peak values across each individual's repeated samples. For the *A. azarae* samples, only five individuals contributed multiple samples from the same gland. In these cases, samples were averaged. Two subadult *A. azarae* were also sampled as juveniles. In these cases, their juvenile samples were not included in calculating average individual values, and were treated as independent juvenile samples. We used transformed peak values to perform stepwise forward variable selection to identify the peaks that separated the groups most for each dependent variable ("greedy.wilks" function in the klaR package in R; Weihs et al., 2005). The peaks selected during the stepwise process were incrementally added as variables in linear discriminant analysis (using the "lda" function in the "MASS" package; Venables & Ripley, 2002). We assessed how well each model

classified individuals into groups by assessing the correctness rate:

Correctness rate = ((correct group 1 classifications)/(n group 1) + (correct group 2 classifications)/(n group 2)) / 2

All of the correctness rates that we report represent the leave-one-out cross-validated classification rate for the models, and refer to the percentage of samples correctly classified. We considered the best models to be those that generated the highest correctness rate with the fewest variables.

Chemical Distances

To evaluate whether relatedness, individual identity, and contraception status are encoded in glandular secretions, we used chemical distances to estimate variation in chemical profiles within and between individuals. Chemical distances (CD) between samples were generated by calculating the Euclidean distance for each possible sample dyad. Smaller values suggest that the chemical profile of the samples within a dyad are more similar, whereas larger values suggest greater differences between samples. Next, we compared the chemical distances between "groups" using the chemical distances generated for all dyads within the following groups: a) males and females to assess sex differences in intra-sexual variation, b) close-kin (parent-offspring or full-sibling dyads) and non-kin (individuals not sharing any grandparents) to evaluate relatedness, c) intra- and inter-individual to test individual identity over time (captive) and across gland type (wild), d) subcaudal and pectoral (wild) to compare variation based on gland type, e) North room and South room (captive) to estimate variation within colony rooms, f) contracepted and non-contracepted females (captive) to evaluate contraception (Table 3

details each comparison, samples used, and dyads excluded from each analysis). Based on our predictions, we expected to find smaller CDs for close-kin than non-kin dyads, and for intra-individual than inter-individual dyads. We also expected to find smaller CDs among contracepted females than non-contracepted females given that they experience less hormonal fluctuation.

Because these data did not satisfy the criteria for assumptions of normality, we used the non-parametric Wilcoxon Rank Sum test to inferentially compare the chemical distances between groups, and we calculated the effect size "r", using the "rFromWilcox" function (Field et al., 2012). As with the classification analyses, we used average relative values of peaks for each individual to calculate CDs, except in the case of inter- and intra-individuals comparisons, in which we used all samples.

Results

We identified 110 peaks endogenous to the subcaudal (N=274) and pectoral samples (N=22) collected from 104 captive *A. nancymaae* individuals and 70 peaks in the subcaudal (N=37) and pectoral (N=35) samples collected from 33 wild *A. azarae* individuals. For both the captive and wild data sets, the total area of the chromatogram, representing the total abundance of compounds detected, was greatest in the subcaudal glands, and lowest in the blank and control vials (Figure 2, Figure 3).

Classification of Glandular Secretions

Male and female glandular secretions in both populations differed chemically. *A. nancymaae* individuals were accurately classified in the LDA model with 89% accuracy

and *A. azarae* individuals were correctly classified by sex 69% of the time (Table 4, Figure 4). Females were more accurately classified than males in both populations (Table 4).

Chemical differences in adult and subadult secretions were more apparent in the *A. azarae* than the *A. nancymaae*, with correctness rates of 76% and 60% respectively (Table 4, Figure 4).

Secretions from pectoral and subcaudal samples of owl monkeys differed markedly in their chemical composition. Samples were classified with 89% and 75% accuracy in the *A. nancymaae* and *A. azarae* populations respectively (Table 4, Figure 4).

Location within the colony (North or South room) was also associated with differences in the chemical profile of *A. nancymaae* subcaudal secretions, with a correctness rate of 81% (Table 4). When this model was used to classify control samples according to the rooms in which they were sampled, control swabs (N=21) were classified correctly only 61% of the time.

Chemical Distances (CDs)

We observed marked sex differences in CD when comparing same sex dyads. The median CD between male-male dyads was greater than that observed in female-female dyads for both *A. nancymaae* subcaudal, *A. azarae* subcaudal, and *A. azarae* pectoral secretions (Table 5). All these differences reached statistical significance, but the magnitude of difference was greater between the sexes in *A. azarae* than in *A. nancymaae*.

Close-kin dyads did not have more similar chemical profiles than non-kin dyads in *A.*

nancymaae and the differences were not statistically significant (Table 5).

Chemical distances of samples from the same individual were smaller than CDs from different individuals in *A. nancymaae* and *A. azarae*. The median CD of intra-individual dyads was less than inter-individual dyads among the *A. nancymaae* subcaudal samples (Table 5). Among the *A. azarae*, the median CD between subcaudal and pectoral samples from the same individual were lower, although not statistically significantly different, than the median CD of subcaudal and pectoral samples from different individuals (Table 5).

We also observed differences in CD based on gland type in the *A. azarae* and housing location in the *A. nancymaae*; these differences reached statistical significance. On the other hand, there were no differences between the medians of females on or off contraception. Among the *A. azarae*, CDs between subcaudal secretions were much larger than CDs between pectoral secretions (Table 5). Captive *A. nancymaae* individuals housed in the North room had more similar chemical profiles than individuals in the South room (Table 5). There were no differences in the median CDs between contracepted and non-contracepted captive *A. nancymaae* females (Table 5).

Discussion

Our study suggests that owl monkey glandular secretions encode biologically relevant information. We found similar patterns in the glandular secretions of two owl monkey species, *A. azarae* and *A. nancymaae*, each in a different environment, wild and captivity. These patterns are positively related to sex, age, individual identity, gland type, and housing, suggesting that information is encoded in glandular secretions, which may act as

chemosignals. The fact that these putative signals were reliably observed in two species, despite the differences in the data sets, speaks strongly of a real phenomenon of biological relevance.

As predicted, there were consistent sex differences in the chemical composition of glandular secretions in both taxa, confirming the chemical dimorphism found in a preliminary study of a smaller population of captive *A. nancymaae* (MacDonald et al., 2008). While an olfactory sex signal in a primarily nocturnal taxon is not surprising in and of itself, it is particularly notable given that there have been virtually no reports in owl monkeys of conspicuous, marked, or seemingly biologically meaningful sex differences in size, body mass, growth development, dispersal patterns, fur coloration (Fernandez-Duque, 2011), and even close inspection of their external genitalia (Spence-Aizenberg et al., submitted). In addition to sex differences in the chemical composition of glandular secretions, we also estimated marked and consistent sex differences in the chemical distances. In both the captive and the wild populations, both the pectoral and subcaudal secretions of female-female dyads were more similar (i.e. had a smaller CD) than those of male-male dyads. This finding suggests that putative chemosignals among male owl monkeys varies more than among females. Given that dimorphism, and variation of the dimorphic trait, are two of the requirements to identify sexually selected traits (Snowdon, 2004), this result supports the hypothesis that traits associated with the production of secretions in owl monkeys may be sexually selected traits, as have been proposed for other primate taxa (Heymann, 2003a; Drea, 2015).

The chemical composition of the glandular secretions varied with age. While the model for age category performed well, with greater than 75% accuracy for the wild

samples – comparable to what has been reported for male mandrills (Setchell et al., 2010; Vaglio et al., 2016) – it did not perform as well, with 60% accuracy, for the captive ones. Given the characteristics of our datasets, the performance of these models highlight the need to reflect on the criteria that our project uses to define age categories. In our analyses we relied on age categories of adult (>48 mos.) and subadult (24.1-48 mos.) that were established considering the age of immigration (approximately four years old) and age at first reproduction (never before four years old) within a wild population of *A. azarae* (Huck et al., 2011). However, this differs from our observations of captive subjects in a related study, in which an *A. nancymaae* breeding pair had an age of first reproduction as early as 38 months (male) and 45 months (female; Spence-Aizenberg et al., unpublished data). The age categories of adult and subadult used by our project are not defined in relation to reproductive development or maturity. Yet, evidence suggests that reproductive function is likely linked to the development and use of the subcaudal gland. For example, immature *Aotus* do not have well-developed subcaudal glands (Hill et al., 1959), but the administration of testosterone to a captive male less than one year old was correlated with an earlier development of this gland (Dixson et al., 1980). In our study, the juvenile and subadult (<48 mos) *A. azarae* samples had a total abundance of chemical compounds in their chromatograms approximately 35% less than in adults, whereas the mean total abundance for the subadult *A. nancymaae* were comparable to adult *A. nancymaae* (7% less total abundance). The lower abundance suggests either a lower amount of secretion produced, and/or a less chemically rich secretion. If glandular development is correlated with rising levels of reproductive hormones, then age categories defined by life history traits in a wild population may not be biologically

relevant in the context of olfactory communication and glandular development. Furthermore, recent research on wild *A. azarae* shows that subadult females exhibit reproductive hormones at levels similar to those of adults (Corley et al., 2017). This, combined with the reproductive success of subadults in captivity, suggests that the captive and wild individuals we categorize as subadults may span a range of reproductive functioning, and highlights a need to reevaluate the criteria used to define age categories.

Owl monkeys apparently have short-term individual signatures of odor. We conclude this based on the similarity of chemical profiles within individuals – over the course of two to three months in the captive population and across pectoral and subcaudal glands within an individual in the wild population – when compared to variation between individuals. Evidence for signals of individual identity in glandular secretions have been found in marmosets (Smith, 2006), ring-tailed lemurs (Scordato et al., 2007), and mandrills (Setchell et al., 2010). An ability to recognize individual identity encoded in odor would be useful in both territory defense and pair bonding. Scent-marks from unfamiliar individuals would signal the presence of extra-group solitary individuals, potentially promoting territory defense. Additionally, the ability to recognize an individual's odor may facilitate the pair bonding process. Odor plays a critical role in pair formation among socially monogamous prairie voles (*Microtus ochrogaster*); the removal of the vomeronasal organ, or the olfactory bulb, diminishes the development of partner preference (Williams et al., 1992; Curtis et al., 2001). In common marmosets, individuals can be conditioned to sexual arousal using an arbitrary odor (Snowdon et al., 2011). It is possible, then, that owl monkeys become familiar with, and conditioned to, the individual odors of the potential partners during the pair formation process, ultimately

facilitating pair bonding.

The secretions produced by the pectoral and subcaudal gland were chemically distinct in both taxa. This is not surprising given that there are marked differences in the frequency with which these glands are used in scent-marking, and that the secretions differ in color and amount, with the pectoral gland secreting a colorless secretion, while the subcaudal gland was typically secreting a dark, oily secretion in much greater amounts (Spence-Aizenberg et al., unpublished data). That individuals sniff the chest of group members but rarely scent-mark with the pectoral gland suggests that it may be used primarily for close-contact communication, likely serving a different function than the subcaudal gland. Our observations parallel those described for ring-tailed lemurs, where different glands are associated with differences in the chemical profiles and color of the glandular secretions (Scordato and Drea, 2007).

There was no evidence for a chemosignal of relatedness. Contrary to our predictions, there were no substantial differences in the overall chemical profile of close-kin and non-kin dyads. Our results also contradict a previous study reporting familial differences in owl monkey odor (MacDonald et al., 2008), although the small number of individuals used in this earlier study represented only three family groups who were also housed together. Therefore, the differences in that study may represent environmental, rather than familial, differences. While we found no evidence of chemosignals of kinship, it may be that some patterns of relatedness in secretions were obscured as we used pedigree, rather than genotype, to estimate relatedness. Pedigree was not found to correlate statistically with chemical distance in mandrills (Setchell et al., 2011), but relatedness based on genotype was found to correlate with chemical distances during the

breeding season in ring-tailed lemurs (Charpentier et al., 2008; Boulet et al., 2009b, 2010). Alternatively, it may be that relatedness may not be as important in mate choice as other genetic components. For instance, chemical distances in mandrill secretions were statistically significantly correlated with MHC dissimilarity (Setchell et al., 2011), and individual heterozygosity is correlated with the diversity of fatty acids in ring-tailed lemur labial secretions (Boulet et al., 2010). Moreover, although chemical analyses have identified volatile compounds associated with MHC type in mice, and mice can behaviorally differentiate between MHC types using urinary odor (Kwak et al., 2008), there is cross-study variation of the volatiles that have been associated with MHC type in mice. It is likely then, that some aspects of odor perception cannot readily be evaluated by chemical measurements of volatile organic compounds even when the behavioral responses to odor variants are robust, as is the case with MHC type in mice (Kwak et al., 2010). Ongoing research to assess the ability of owl monkeys to perceive relatedness through olfactory cues (Chapters 2 and 4) will provide additional insights into the possible role of kinship recognition in regulating olfactory communication in owl monkeys.

There were mixed influences of housing and management on the chemical profile of captive individuals. Contraception had little to no effect on the odor of females, whereas location within the colony had a profound effect. Increased similarity in the chemical profiles of females receiving contraception would indicate that it altered the chemical profile so that there would be convergence among contracepted females, as has been reported for ring-tailed lemurs (Crawford et al., 2011). Surprisingly, the negligible differences in chemical profiles between non-contracepted and contracepted *A.*

nancymaae females suggest contraception does not much alter the overall chemical composition of subcaudal glandular secretions, despite the expected hormonal differences in females receiving contraception. Additionally, contraception does not impede the ability of females to form new pairs with males (L. Williams, personal communication), suggesting that the volatile metabolome was not drastically altered. However, within individual comparisons would improve the robusticity of these results.

The important chemical differences between samples from individuals housed in different colony rooms merit explanation. The most likely cause is environmental as there are no obvious sex or age differences in the animals sampled from these two rooms. Other environmental factors, including the standard diet and cleaning protocols, were the same in both rooms, and ambient environment is unlikely the cause as the control samples collected in each room could not be discriminated based on location. Therefore, the most evident environmental difference is dietary, as one room was receiving a diet supplemented with peanut butter while the other room did not. Given that the diet, and protein sources in particular, can influence body odor (Ferkin et al., 1997; Havlicek and Lenochova, 2006), the dietary peanut butter supplements are the most plausible explanation for the chemical differences between animals in these two locations. Some of the compounds tentatively identified likely derived from diet. Specifically, 2-pentyl-furan – the identity of one of the compounds in the model for location – is not known to derive from mammalian metabolism and likely derives from diet according to the Pubchem online database (National Center for Biotechnology Information., CID=19602).

When comparing results across species and contexts, we found that the models tended to less accurately classify wild *A. azarae* than captive *A. nancymaae*. While it is possible

this is due to species differences, it seems more likely that differences in environment, sample handling, and data analysis contributed to increased variability in the *A. azarae* samples, reducing the ability to discriminate biologically meaningful variables. For instance, individuals in the wild have greater variation in diet both between groups (van der Heide et al., 2012) and throughout the year (Fernandez-Duque et al., 2002). Additionally, samples collected in the field were not maintained continuously at freezing temperatures until arrival to the laboratory in the United States; changes in temperature are associated with a loss of volatiles in other taxa (Hayes et al., 2006; Drea et al., 2013). A potential loss of volatiles may be the reason for our finding that the samples from captive individuals were chemically richer than those from wild ones, with approximately 1.5 times the number of endogenous peaks. Finally, there were fewer wild individuals sampled than captive ones, which meant that we had to pool subcaudal and pectoral secretions, making it more difficult to identify other traits potentially causing variation in odor. Differences between the performances of models notwithstanding, the similarity in many of the results reinforces the notion that there are biologically meaningful patterns in the data.

In summary, it is hardly surprising that owl monkey odors encode information given the nocturnal habits of the taxon, the near absence of sexual dimorphism in physical features, and the frequency with which they engage in olfactory social behaviors. In both the captive *A. nancymaae* and wild *A. azarae* samples we found evidence for putative signals reported in other non-human primate taxa, including sex, age, individual identity, and gland type, but not for relatedness, nor contraception status.

We have identified volatile compounds as putative signals in glandular secretions of

owl monkeys, but this is only one component of the study of olfactory communication. Without confirming that these putative signals are perceived, we cannot identify them as chemosignals. Our ongoing implementation of behavioral bioassays and behavioral, hormonal, and olfactory monitoring of breeding pairs will complement the research presented here by addressing other facets of olfactory communication in *Aotus*. Beyond this, future work incorporating genetic measures of relatedness, non-volatile chemical cues in glandular secretions and urine, coupled with a better understanding of mate choice and the pair formation process, will surely contribute to a more comprehensive understanding of the role of olfactory communication in forming and maintaining male-female relationships, and how these processes may differ from non-monogamous taxa.

Tables

Table 1: Number of male and female individuals in the captive *A. nancymaae* and wild *A. azarae* populations from which subcaudal and pectoral gland secretion samples were collected

		Captive Individuals		Wild Individuals	
Sex	**Age**	**Subcaudal**	**Pectoral**	**Subcaudal**	**Pectoral**
Female	*Adult*	39	10	6	6
	Subadult	13	3	7	7
	Juvenile	--	--	1	2*
	unknown	--	--	1	1
Male	*Adult*	33	5	8	11
	Subadult	19	4	4	3
	Juvenile	--	--	2*	2*
	TOTAL	**104**	**22**	**29**	**32**

* one juvenile was also sampled as a subadult

Table 2: Peak ID, retention time, compound identification and spectral match certainty of identification (between parentheses) for peaks used in LDA models for samples of captive *A. nancymaae* and wild *A. azarae*. Compounds in bold were positively identified using standards (see Appendix 1)

Species	Peak	Retention Time (min)	Model	Identified compound (%)
A. nancymaae	598	6.0	Location	2-Pentanone (90)
	667	6.4	Age Category	Unknown
	1053	8.3	Sex	**4-Heptanone**
	1085	8.5	Gland Type, Location	Unknown
	1297	9.6	Gland Type	**2-Heptanone**
	1448	10.3	Location	**2-Pentyl-furan**
	1865	12.5	Age Category	**4-Nonanone**
	2453	15.4	Sex	Unknown
	2507	15.7	Location	Unknown
	2718	16.8	Age Category	**Benzaldehyde**
	2764	17.0	Gland Type	4-Acetyl-1-methylcyclohexene
	3473	20.6	Sex	Azulene* (36)
	3887	22.7	Age Category	trans-Shisool (30)
A. azarae	1392	10.1	Gland Type	**1-Butanol**
	1674	11.5	Gland Type	2,3,3-trimethyl-Cyclobutanone (48)
	2379	15.1	Age Category	Unknown
	2713	16.7	Sex	Unknown
	2977	18.1	Age Category	**Linalool**
	3867	22.6	Age Category	1-(2-butoxyethyoxy)-ethanol (49)
	4892	27.78	Gland Type	5-Isoxazolecarboxylic acid (53)
	4964	28.15	Age Category	**4-Ethyl-phenol**

*The likelihood that this peak is azulene is likely much higher, as the NIST Library identified this peak as azulene or naphthalene, and naphthalene was ruled out as the compound at this peak (see Appendix 1)

Table 3: Description of samples included and dyads excluded from all chemical distance analyses. Results of the comparisons between chemical distances are in Table 5

Species	Dyad Comparison	Sample Type(s)	Excluded from analyses
A. nancymaae	M-M vs. F-F	SC-SC	M-F dyads; intra-individual dyads
	Close-Kin vs. Non-Kin	SC-SC	intra-individual dyads; individuals not associated with a family group
	Intra- vs. Inter-Individual	SC-SC	M-F dyads
	North vs. South Room	SC-SC	intra-individual dyads
	Non-* vs Contracepted Fs	SC-SC	intra-individual dyads
	All Dyads	SC-SC	none
A. azarae	M-M vs. F-F	SC-SC	M-F dyads; intra-individual dyads
	M-M vs. F-F	PE-PE	M-F dyads; intra-individual dyads
	Intra- vs. Inter-Individual	SC-PE	M-F dyads
	Subcaudal vs. Pectoral	SC-SC, PE- PE	intra-individual dyads
	All Dyads	SC-SC, SC-PE, PE-PE	none

*Non-: non-contracepted females; SC-SC: subcaudal-subcaudal sample dyads; SC-PE: subcaudal-pectoral sample dyads; PE-PE: pectoral-pectoral sample dyads; M-M: male-male sample dyads; F-F: female-female sample dyads; Fs: Females

Table 4: Peaks included in the best performing Linear Discriminant Analysis model, correctness rate, and classification summary of glandular secretions from the subcaudal and pectoral samples obtained from captive *A. nancymaae* and wild *A. azarae*.

Species	Category (Sample Type)	Peaks Included*	Correct-ness Rate	Correctly assigned (group type)	Incorrectly assigned (group type)
A. nancymaae	Sex (SC)	1053, 2453, 3473	89%	51 (females) 42 (males)	1 (females) 10 (males)
	Age (SC)	1865, 667, 3887, 2718	60%	64 (adults) 10 (subadults)	8 (adults) 22 (subadults)
	Gland Type (SC, PE)	1085, 1297, 2764	89%	101 (SC) 18 (PE)	3 (SC) 4 (PE)
	Location (SC)	1085, 598, 1448, 2507	81%	37 (North room) 32 (South room)	6 (North room) 10 (South room)
A. azarae	Sex (SC & PE)	2713	69%	23 (females) 19 (males)	8 (females) 11 (males)
	Age** (SC & PE)	4964, 3867, 2379, 2977	76%	28 (adult) 13 (subadult)	3 (adult) 8 (subadult)
	Gland Type (SC & PE)	1674, 4892, 1392	75%	21 (SC) 25 (PE)	8 (SC) 7 (PE)

SC: subcaudal, PE: pectoral; *see Table 2 for tentative identity of each peak; **excluding wild juveniles

Table 5: Medians, effect sizes, and statistical tests of differences in chemical distances of subcaudal secretion samples in captive *A. nancymaae* dyads and subcaudal and pectoral secretions samples in wild *A. azarae* dyads

Species	Dyad Compar-ison	Median Euclidean Distance (Range)	N dyad	Effect Size (r)	Wilcoxon Rank Sum (W)	P Value
A. nancymaae	M-M vs. F-F	M-M: 0.24 (0.08-0.71) F-F: 0.22 (0.09-0.45)	1275 1378	-0.131	745050	<0.001
	Close-Kin vs. Non-Kin	Close-kin: 0.23 (0.11-0.64) Non-kin: 0.23 (0.08-0.70)	164 2466	-0.020	211770	0.31
	Intra- vs. Inter-Individual	Intra-: 0.29 (0.13-0.67) Inter-: 0.31 (0.08-0.84)	195 15262	-0.025	1657400	<0.01
	North vs. South Room	North: 0.19 (0.08-0.37) South: 0.25 (0.12-0.71)	903 861	-.436	192700	<0.001
	Non-* vs Contra-cepted Fs	Non-: 0.23 (0.09-0.45) Contra-: 0.23 (0.13-0.37)	277 120	-0.014	16337	0.79
	All Dyads	0.32 (0.08-0.84)	5356	n/a	n/a	n/a
A. azarae	M-M vs. F-F (SC)	M-M: 0.54 (0.15-0.97) F-F: 0.23 (0.08-0.89)	90 105	-0.434	2345	<0.001
	M-M vs. F-F (PE)	M-M: 0.25 (0.06-0.76) F-F: 0.16 (0.05-0.78)	119 119	-0.286	4734	<0.001
	Intra- vs. Inter-Individual	Intra-: 0.33 (0.09-0.89) Inter-: 0.35 (0.06-1.02)	26 435	-0.016	5887	0.726
	Subcaudal vs. Pectoral	SC: 0.49 (0.07-1.00) PE: 0.21 (0.04-0.88)	405 494	-0.394	54293	<0.001
	All Dyads	0.33 (0.04-1.02)	1830	n/a	n/a	n/a

*Non-: non-contracepted females; M-M: male-male sample dyads; F-F: female-female sample dyads; Fs: Females; SC: subcaudal dyads; PE: pectoral dyads

Figures

Figure 1: The subcaudal gland of a captive male (a) and female (b) *A. nancymaae*

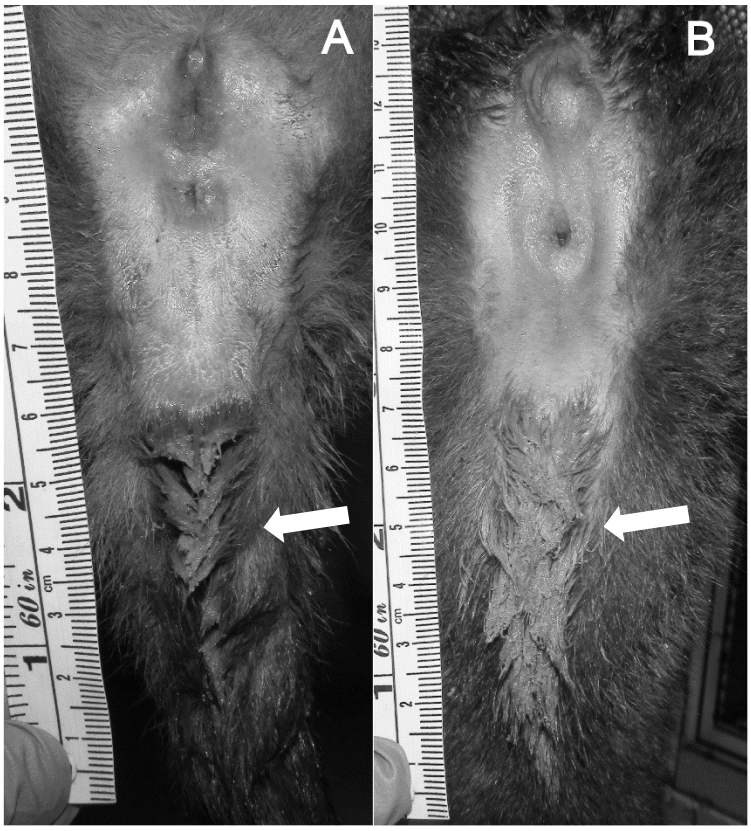


Figure 2: Chromatogram of a blank vial (top), and glandular secretion from the pectoral gland (middle) and subcaudal gland (bottom) of a captive adult female *A. nancymaae*

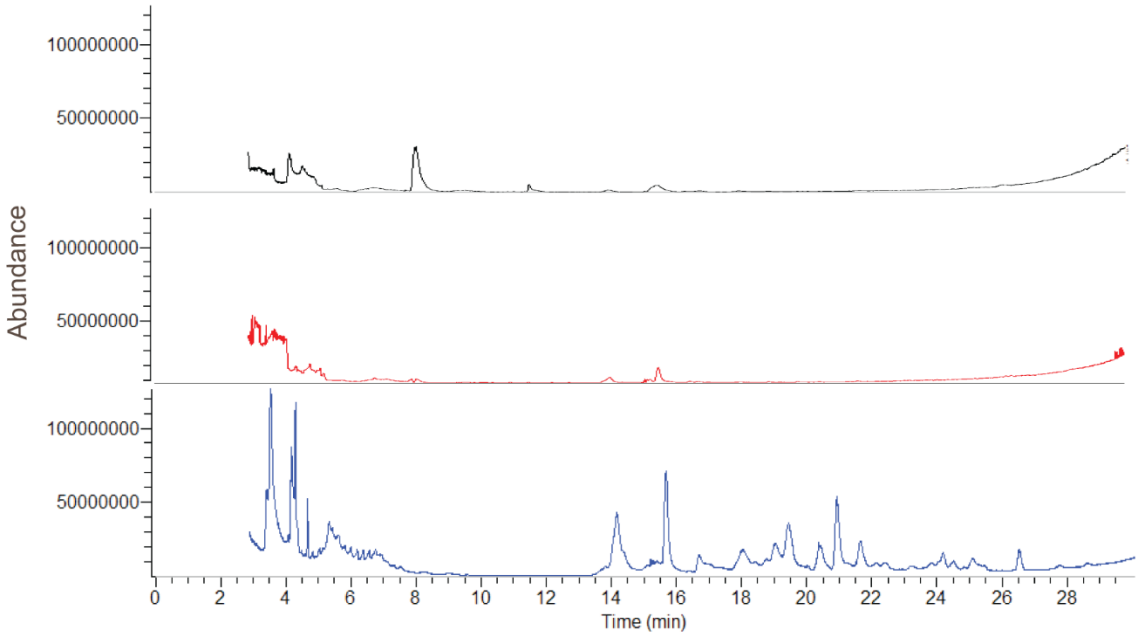


Figure 3: Mean values for the total chromatogram area in blank/control vials, pectoral secretions, and subcaudal secretions from the captive and wild datasets. Error bars represent the SEM

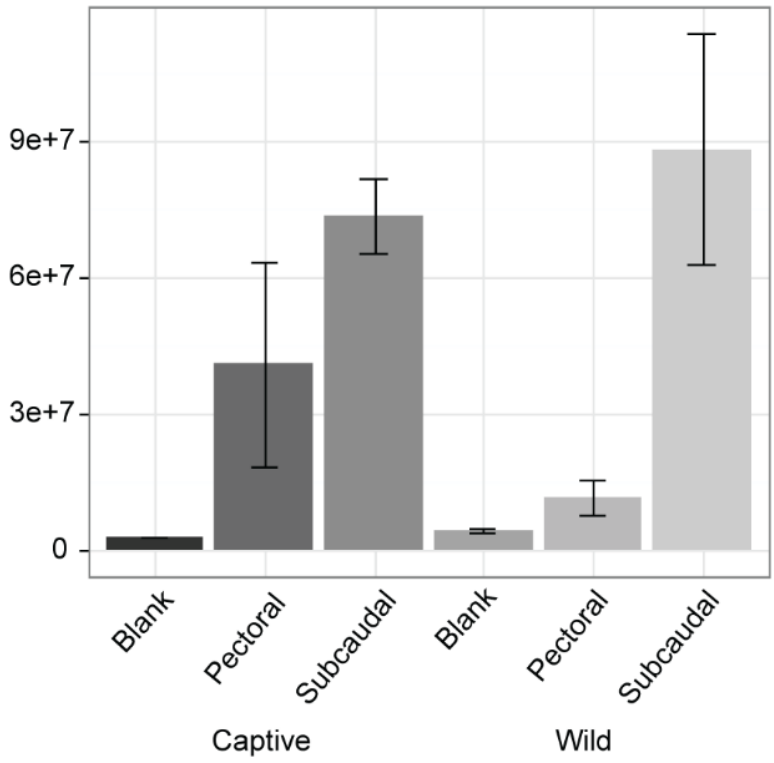
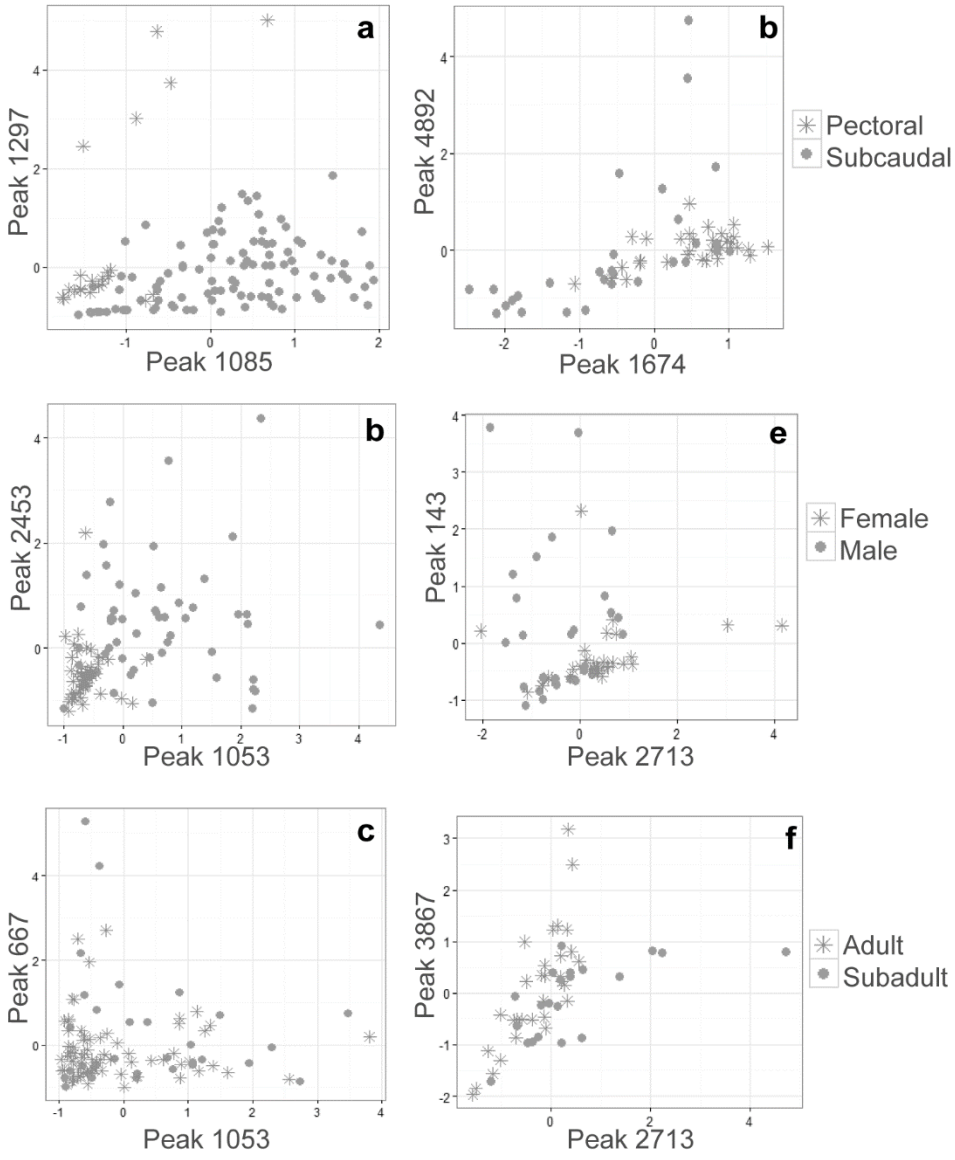


Figure 4: Individual averages of square-root transformed and scaled relative peak values for the first two peaks in the LDA model to discriminate captive *A. nancymaae* by a) gland type, b) sex, and c) age category, and wild *A. azarae* by d) gland type, e) sex, and f) age category



CHAPTER 3: Discrimination of kin and preference for inbreeding in a pair-bonded socially monogamous primate

Abstract

Owl monkey glandular secretions are chemically rich and encode information that could be used to signal to potential mates. Given the long-term bonds and infrequent opportunities for extra-pair paternity, cues of partner quality, such as relatedness, may be particularly important. We propose that owl monkeys use chemical signals of relatedness when forming a new pair. To investigate this, we conducted behavioral bioassays in a captive population of *A. nancymaae*, to evaluate whether owl monkeys can discriminate between unfamiliar full-siblings or unfamiliar non-kin using olfactory cues alone. Next, using microsatellites we looked at estimates of genetic relatedness between wild *A. azarae* pairs, and compared these to simulations of random mating within the population to see if relatedness between pairs was more similar to inbreeding avoidance, inbreeding preference, or random mating. We found that owl monkeys, overall, spend more time sniffing the odor of full-siblings, although there was substantial variation across trials. We also found that wild pairs show a much higher mean relatedness between partners than if mating were random, suggesting that some individuals prefer to mate with close kin. These results are consistent with the hypothesis that owl monkeys can use odor to discriminate between individuals based on relatedness, and cues of relatedness are used in mate choice. This is the first evidence of kin discrimination in owl monkeys and of inbreeding preference in a wild primate population.

Introduction

The ability to discriminate between related and unrelated individuals can serve many adaptive functions, including influencing mate choice, mediating parent-offspring interactions, and facilitating nepotism. Much of the research on kin recognition spurred by Hamilton's (1964) theory of inclusive fitness focused on the ability of group-living organisms to discriminate between individuals based on relatedness, and bias their behavior accordingly (Holmes, 2004). Numerous studies, particularly in group living organisms, have since attempted to demonstrate kin discrimination abilities and the direction of nepotistic behavior according to relatedness (Silk, 2002; Mateo, 2003; Holmes, 2004; Widdig, 2007).

However, the function of kin discrimination may differ in group-living and pair-living, or socially monogamous, taxa. The primary relationships for these individuals are likely with their social and/or sexual partner, potential mates, and offspring. Thus, when considering kin recognition in socially monogamous animals, it is arguably most relevant in relationships between (potential) mates, and parents and offspring. This idea is supported by patterns of individual recognition in geladas (*Theropithecus gelada).* Gelada males have limited vocal recognition, with males vocally recognizing other males in their unit but not males outside of their unit even though they may interact regularly with them (Bergman, 2010). This suggests that the ability to recognize individuals may be limited to those individuals that are most important to recognize.

In the context of mate choice, kin recognition would make it possible for individuals to actively avoid or prefer mating with close relatives (Pusey and Wolf, 1996; Lehmann and Perrin, 2003). Additionally, the ability to recognize genetic kin would

enable individuals to recognize and limit care towards their own offspring and avoid inbreeding depression. Mate choice in socially monogamous taxa may be even more important than in taxa with different mating systems, particularly if breeding relationships are long-term and individuals have more limited mates within a lifetime. In such cases, mate choice will have an influence beyond the current breeding season, and the degree of relatedness between partners could have a significant impact on the reproductive success of the pair. Offspring of close relatives may experience inbreeding depression, decreased fitness compared to offspring from less related individuals, which may affect their survival and reproduction (Keller and Waller, 2002; Charpentier et al., 2007). The avoidance of inbreeding may improve an individual's overall fitness, particularly when inbreeding depression is strong, and kin recognition is one possible mechanism to achieve this goal (Pusey and Wolf, 1996). Not reproducing with close kin when close kin are available as mates suggests that kin discrimination plays a role in mate choice. For example, the Australian sleepy lizard (*Tiliqua rugosa*) form monogamous pairs, and partners are less closely related to each other than expected by chance. Similarly, pair-living White's skink (*Liopholis whitii*) can discriminate close kin from non-kin (Bordogna et al., 2016). While their social partner is more closely related to them than if mating were random, extra-pair mates are less related than expected if matings within the population were random (While et al., 2014). This observation suggests that these lizards and skinks use some degree of kin recognition in mate choice, possibly to avoid inbreeding depression.

On the other hand, kin recognition could also facilitate inbreeding preference. Theoretically, inbreeding may be favored in some circumstances, even when inbreeding

depression is present, if the inclusive fitness benefits exceed the costs associated with this strategy (Lehmann and Perrin, 2003; Kokko and Ots, 2006; Puurtinen, 2011; Duthie and Reid, 2016). The circumstances favoring inbreeding preference may differ for monogamous and polygynous mating systems (Lehmann and Perrin, 2003; Lehtonen and Kokko, 2015). Kokko & Ots (2006) detail three criteria that may increase the inbreeding threshold in a population: 1) both sexes invest heavily in infant care; 2) encounter rates for a mate are low; and 3) mating is sequential rather than simultaneous, conditions that are more likely for pair-living than group-living organisms.

There is empirical evidence indicating that some socially monogamous taxa exhibit inbreeding preferences. The African cichlid fish (*Pelvicachromis taeniatus*), a socially monogamous fish with biparental care (Thünken et al., 2010), can discriminate between opposite sex siblings and non-siblings through chemical cues (Thünken et al., 2014), and preferentially mate with unfamiliar siblings, siblings they were not reared with, in an experimental setting (Thünken et al., 2007). Additionally, among ground tits (*Parus humilis*) (Wang and Lu, 2011) and barn swallows (*Hirundo rustica erythrogaster*) (Kleven and Jacobsen, 2005), females in socially monogamous pairs were more closely related to their extra-pair mates than expected by chance. These examples suggest some degree of inbreeding preference as well as a mechanism of kin discrimination.

Recognition of genetic offspring could be particularly beneficial to males in taxa where males provide care for infants and the costs of caring for unrelated infants are high. Yet, examples of this kind of recognition are extremely limited (Neff and Sherman, 2005), and often absent in taxa with high rates of extra-pair copulations, where males have the greatest risk of misallocating care (Kempenaers and Sheldon, 1996). In such

cases where promiscuity leads parents to risk misallocating care, the absence of kin discrimination might be maintained by a signaler-recipient conflict (Beecher, 1991), if a signal of relatedness would lead the offspring to lose out on care. However, in cases where the likelihood of misallocating care is low, such as when males and females do not seek extra-pair copulations, there may be no benefits to recognizing genetic offspring.

Olfaction is a likely mechanism for kin recognition in primates. The ability to identify genetic components, such as MHC type, through body odor (Yamazaki and Beauchamp, 2007; Kwak et al., 2008) and to use this information in mate choice (Yamazaki and Beauchamp, 2007) has been well established in mice. Among primates, there is growing evidence that genetic differences in MHC type (Knapp et al., 2006; Setchell et al., 2011), heterozygosity and genetic relatedness (Charpentier et al., 2008, 2010; Boulet et al., 2009a) are present in glandular secretions. Furthermore, primates are able to discriminate between odors based on these genetic differences (Wedekind et al., 1995; Jacob et al., 2002; Charpentier et al., 2010), although evidence of whether this extends to preferences in mate choice is mixed (Winternitz et al., 2017).

We investigated whether there is evidence of kin recognition in a pair-living non-human primate, the owl monkey (*Aotus* spp.). Owl monkeys form long-term reproductive pair bonds spanning multiple breeding seasons, with approximately two mates over a lifetime (Fernandez-Duque and Huck, 2013). There is no evidence of extra-pair paternity in a wild population (Huck et al., 2014), suggesting that extra-pair copulations are a rare or unsuccessful strategy to secure matings. Male owl monkeys contribute heavily to infant care, providing the vast majority of infant carrying, food sharing, and playing (Dixson and Fleming, 1981; Wright, 1984; Rotundo et al., 2005; Huck and Fernandez-

Duque, 2012). Step-fathers, i.e., immigrant males who replace the resident male, provide care to infants present in the group at the time of their takeover (Fernandez-Duque et al., 2008). This combination of monogamy and biparental care make owl monkeys a valuable model to test the kin recognition hypothesis, although our interest in this study centers primarily on the role that kin recognition may play in mate choice.

We hypothesized that owl monkeys use chemosignals to recognize kin and select mates. If owl monkeys can discriminate kin using chemical signals, then we predicted that they differentially attend to odors from close kin (defined here as parent, offspring, or full sibling), and non-kin (defined as not sharing any parents or grandparents). If cues of kinship are used in mate choice, we predicted that estimates of relatedness between social pairs in a wild population are lower (suggesting some degree of inbreeding avoidance) or greater (suggesting some degree of inbreeding preference) than expected if pairs formed randomly. No difference in relatedness between social partners and random mating simulations would suggest mating is random, and kin recognition is not relevant to mate choice in wild owl monkeys.

Furthermore, because sex-biased dispersal is also a mechanism for inbreeding avoidance (Pusey and Wolf, 1996), we considered the potential role sex-biased dispersal may play in our wild population of owl monkeys. Males and females both disperse from their natal group, and sibling encounters in reproductive groups have been observed (Fernandez-Duque, 2009). Therefore, we predicted there is no sex-biased dispersal, and thus no sex differences in relatedness between male-male and female-female dyads. Additionally, although not directly related to our hypothesis, we considered whether there are sex differences in preference for close kin or non-kin, given that male owl monkeys

investigate olfactory cues more frequently than females (Spence-Aizenberg, Chapter 2). Finally, we also conducted control bioassays to establish that owl monkeys can detect odors of conspecifics in the testing paradigm.

To test our predictions, we coupled an experimental approach in captive Nancy Ma's owl monkeys (*A. nancymaae)* with an observational study of wild Azara's owl monkeys (*A. azarae)*. Behavioral bioassays in captivity allow us to experimentally identify chemosignals in ways that cannot be accomplished in the field, largely due to the neophobic behavior of the owl monkeys (Fernandez-Duque, personal communication), and assess the ability of owl monkeys to discriminate individuals based on a pedigree estimate of relatedness. Complementing this research with observations of naturally occurring social pairs and reproductive behavior in a wild population allows us to learn about the potentially adaptive value of the putative chemosignals across *Aotidae*, and to infer kin recognition based on their reproductive choices.

Methods

Behavioral Bioassays

Subjects

For the behavioral bioassays, we worked with a captive population of *Aotus nancymaae* housed at the Owl Monkey Breeding and Research Resource (OMBRR) located in the Keeling Center for Comparative Medicine and Research (MD Anderson Cancer Center, University of Texas, Bastrop). The OMBRR houses approximately 400 owl monkeys in two large colony rooms and has a semi-reversed light cycle with periods of darkness extending approximately from 1500h to 0000h. Individuals are housed in

family groups or pairs (in enclosures 1.8m^3 in size), or alone when socially required. Individuals receive the same diet: LabDiet® Fiber-Plus® Monkey Diet 5049 (LabDiet; St. Louis, MO) with fruit or vegetable, and water is always available. Enclosures are directly adjacent to one another, but groups are visually isolated from each other and white noise (a waterfall) buffers the acoustic interactions within the rooms. Groups may be within olfactory range of their neighbors, but only in direct contact with their cagemates. All adult females in this study were administered monthly intra-muscular injections of a hormonal contraception, medroxyprogesterone acetate (MPA).

Experimental Design

We conducted a series of choice trials, presenting subjects with two different odorants composed of glandular secretions from other owl monkeys (scent donors) or control odors (a cotton swab). Scent donors (n= 31 males, 25 females) were individuals of the opposite-sex from the subject and "unfamiliar," the subject having never shared a living space with them. Scent donors were classified as either "close-kin" (full siblings, sharing both mother and father with the subject) or "non-kin" (not sharing any maternal or paternal grandparents with the subject). Because we could not control for female reproductive phase, male owl monkeys differentially attend to female odor across the ovarian cycle (Chapter 4), and female performance in bioassays is affected by reproductive state in lemurs (Scordato and Drea, 2007), we only used female scent donors and female subjects that were receiving contraception (Medroxyprogesterone, 150 mg/ml), which suppresses ovulation. Control odors were created by rubbing sterile cotton swabs on the testing surface.

We used two different bioassay testing paradigms: conspecific and kin discrimination trials. In conspecific trials, we presented subjects with the choice between a control odor and the odor of a non-kin scent-donor. These trials were designed to ensure that the monkeys respond more strongly to glandular secretions from conspecifics than to any odor produced by the applicator or the device on which the secretions are presented. In kin discrimination trials, we presented subjects with the choice between a close-kin scent-donor and non-kin scent-donor. These trials were designed to assess whether owl monkeys discriminate between the glandular secretions of individuals based on their degree of relatedness. Close-kin and non-kin scent donors were matched for sex, age (average age difference = 1.2 years), and room location within the colony (North or South Room), due to the influence of these variables on the chemical profiles (Spence-Aizenberg et al., accepted). They were also matched for the type of social group in which they lived (male-female, family, female-female, male-male). Using the effect size observed in some preliminary conspecific trials conducted with *A. vociferans*, we used G-Power (Faul et al., 2009) to determine the sample size needed for the conspecific trials in *A. nancymaae*. We conducted 14 conspecific trials (n=8 males, n=6 females), and 45 kin discrimination trials (n=15 males, n=17 females, number of trials per subject:1-3). Although some subjects were tested more than once, we considered the trials to be independent because all trials were unique as the scent-donors–subject triad were never replicated.

Odorants were presented on "stimulus tubes" (Figure 1), small PVC tubes approximately 5 cm in length and 2.5 cm in diameter. The tubes were reconfigured enrichment feeders, made of a material found throughout their housing, on which they

frequently scent mark and inspect the marks of others. The position of the odorants on the stimulus tubes (left or right, top or bottom) was alternated across trials so that the position of each odor type (non-kin vs. control or close-kin vs. non-kin) was balanced across subjects and trials. Trials in which the subject did not approach both odors (conspecific: N=2, kin discrimination: N=15) were excluded from comparisons.

Prior to the start of each trial, scent-donor samples were brought to room temperature. At least 30 minutes prior to the trial, we removed from the cage the cagemate(s) of subjects who were not individually housed to allow the subject to habituate to the temporary isolation. Immediately before the trial began, we rubbed the control and/or scent-donor swabs on the center of the stimulus tubes, covering approximately 6.5cm^2 (Scordato and Drea, 2007). We then placed these tubes in the subject's cage 25cm apart (Charpentier et al., 2010) (Figure 2).

Data Collection

The trial began when we closed the cage door after the stimulus tubes were hung, and continued for 10 minutes after the subject first approached a stimulus tube within 6 cm. If the subject did not approach a stimulus tube within the first five minutes, the test was continued for an additional 10 minutes and then terminated. We digitally recorded all trials using an infrared HD Sony camera. An infrared lamp provided additional lighting. We played back recordings of trials using Avidemux2.6 (http://www.fosshub.com/Avidemux.html). We recorded all occurrences of interactions with the stimulus tube (Table 1, Figure 2) throughout the entire trial and entered these into the program JWatcher (v1.0/1.1, http://www.jwatcher.ucla.edu/). We also recorded

start and end times (to the millisecond) for each behavioral state (Table 1) and the time of behavioral events.

Data Analysis

For conspecific and kin discrimination trials, we recorded both the frequency and duration of behaviors directed to the stimulus tubes (Table 1), but limited all statistical analyses to the durations of behaviors because the frequency and duration were highly correlated for approach and proximity (Spearman's rank correlation rho= 0.88, S= 272.61, P=<0.001), sniffing (Spearman's rank correlation rho=0.93, S=156.13, P<0.001), and touching (Spearman's rank correlation rho= 0.91, S= 213.79, P=<0.001). We evaluated each of these behaviors separately because they are qualitatively different interactions with the odorants. Licking, open mouth, and scent-marking were observed infrequently, or not at all, and all such instances are reported.

To assess whether owl monkeys can detect odors in the bioassay paradigm, we compared the duration of behaviors directed to the conspecific and control odors within conspecific trials using Wilcoxon signed rank tests. To evaluate differences in the responses of individuals to close-kin and non-kin based odor cues, we conducted Wilcoxon signed rank tests for the time spent in proximity, sniffing, and touching the close-kin and non-kin odors within a trial. We also looked for sex differences in odor preference (the proportion of time an individual directed a behavior to the close-kin odor, out of the total time directed to both odors) using Wilcoxon rank sum tests. For all Wilcoxon tests, we calculated the effect size "r", using the "rFromWilcox" function (Field et al., 2012).

To evaluate inter-observer reliability in scoring the trials, two observers scored 20% of kin discrimination trials (9/46). Agreement rates for each behavior were calculated using this formula:

> # agreements / ((observer 1 #agreements + disagreements + omissions) + (observer 2 # agreements + disagreements + omissions)) / 2 (Coelho and Bramblett, 1981)

For the 9 trials combined, there were 380 agreements, 7 disagreements, 12 omissions (observer 1), and 24 omissions (observer 2). Agreement for individual behaviors were as follows: approach (99%, n=87), sniff (92%, n=139), touch (94%, n=62), open mouth (0%, n=4). We also measured inter-observer reliability by calculating ratios for behaviors directed to the left versus the right stimulus tubes. For the nine trials scored by both observers, we calculated the ratio of the number of approaches, sniffs, and touches, and time spent in proximity, sniffing, and touching the right and the left tubes. We estimated the relationship of these ratios between observers using Pearson's correlation coefficient using "cor.test" in R, and found the ratios between observers to be highly correlated ($r=0.98$) and to deviate from zero (Pearson's product moment correlation coefficient: $t=40.7$, $df=52$, $p<2.2e-16$, 95% C.I. 0.97-0.99). Although concordance rates between observers were high, we used scores from Observer 1 for all conspecific trials, and Observer 2 for all kin discrimination trials to limit any inter-observer variability within each trial type. We conducted all statistical analyses in R (R Development Core Team, 2016).

Microsatellite Relatedness Estimates

Subjects

For estimates of relatedness in partners, we studied a wild population of *Aotus azarae* ranging in gallery forests along the Pilagá and Guaycolec Rivers in Formosa, Argentina (58° 11′W, 25° 58′S). These groups have been regularly monitored since 1997 as part of the Owl Monkey Project, and demographic data from 18 groups have been collected to date. We regularly identify individuals by fitting them with VHF radiocollars, or ball-chain collars with colored beads, after darting and anesthetizing them using ketamine hydrochloride projected from a CO_2-powered rifle and fitted with, following established methods (Fernandez-Duque and Rotundo, 2003; Juarez et al., 2011). We only used pairs in which individuals were positively identified.

Data Collection

Genetic samples (blood, tissue, hair) were collected and genotyped from 124 *A. azarae* in this population in Argentina, and high quality DNA has been extracted from these samples and screened for 14 loci with polymorphic short tandem repeats (Babb et al., 2011). These microsatellites are good candidates to estimate pair-wise relatedness among dyads (Babb et al., 2011; Huck et al., 2014). With these data, and long-term demographic observations of social groups in this population, Huck et al (2014) used CERVUS and Bayesian analysis to identify 61 parent-offspring, 17 full-sibling, and 6 half-sibling dyads (Huck et al., 2014).

Data Analysis

We generated maximum likelihood estimates of the coefficient of relatedness and the most likely relationship (MLR) for all *A. azarae* dyads using ML-RELATE (Kalinowski et al., 2006), following Costello et al. (2008). We treated the coefficient of relatedness as a continuous variable and MLR as a categorical one. To evaluate the accuracy of the ML-Relate output, we compared the coefficient of relatedness and MLRs for the known parent-offspring, full-sibling, and half-sibling dyads identified in Huck et al. (2014).

We limited the remainder of the analyses to dyads of individuals who were in the pool of potential mates. To create this list of dyads, we first considered all individuals in our study to be within the same mating population, as individuals could potentially travel between the territories relatively easily. Second, we only included dispersed individuals (i.e., individuals no longer residing in their natal group) in the pool of potential mates. Third, we only included dyads where both individuals were observed as dispersed adults within the same calendar year, from 1997-2013. We used calendar years as the interval because, although owl monkeys are seasonal breeders, resident males and females have been observed to be replaced throughout the calendar year. Considering these variables, we created a list of 911 male-male dyads, 804 female-female dyads, and 1751 male-female dyads (potential partners). Of these, 42 were observed belonging to the same social group (social partners), and 16 were genetically confirmed to share offspring (genetic partners) (Huck et al., 2014).

To evaluate whether individuals prefer or avoid partnering with close kin, we compared the coefficient of relatedness and MLR classifications between social partners (n=42) and all potential partners (n=1751). Lower values of relatedness or greater

classifications of unrelated dyads among social partners than potential partners would suggest individuals actively avoid partnering with close-kin, whereas similar values would suggest forming a pair is close to random, and higher values would suggest a preference for pairing with close kin. We also compared the coefficient of relatedness and MLRs between social partners (n=42) and genetic partners (n=16) to see if individuals avoided reproducing, if not pairing, with close-kin. We report median values for the coefficients of relatedness as the data were not normally distributed. For all statistical comparisons between dyad types of the coefficient of relatedness, we used Wilcoxon rank sum tests and "rFromWilcox" (Field et al., 2012) to calculate the effect size. For all statistical comparisons of the MLR between dyad types, we used Chi-squared tests.

We also conducted randomization tests following Huchard et al. (2013, 2017) to simulate a distribution of the mean coefficients of relatedness if mating were random, in order to compare this value to the observed relatedness between social partners. Because individuals cannot pair and reproduce without a territory, we did not randomly select dyads. Instead, we generated potential pairings for all male and female residents in a group. For each resident male represented in our group of social partners, we randomly selected 10,000 mates from the pool of available females in the population during the male's tenure. If a male was represented more than once in the list of social partners, we generated sets of 10,000 mates equivalent to their representation. We then calculated mean coefficient of relatedness values for each set of 42 randomly generated mate dyads, creating a total of 10,000 means for males. We repeated the process for females. Next, we compared the distribution of these means to the observed mean coefficient of relatedness for social partners. A two-tailed p-value was calculated as the proportion of simulated

means exceeding the observed mean of social partners on either side of the simulated distribution.

To test for evidence of sex-biased natal dispersal, we compared the same-sex dyads in the pool of potential mates. Sex differences in estimates of relatedness would suggest there is sex-biased dispersal, with the dispersing sex showing lower estimates of relatedness than the non-dispersing sex.

Ethics Statement

The research on the captive *A. nancymaae* individuals was approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee (ACUF# 05-13-04881). The Owl Monkey Project has had continued approval for all research on *A. azarae* presented here by the Formosa Province Council of Veterinarian Doctors, the Directorate of Wildlife, the Subsecretary of Ecology and Natural Resources and the Ministry of Production. At the national level, the procedures were approved by the National Wildlife Directorate in Argentina and by the IACUC committees of the Zoological Society of San Diego (2000–2005) and of the University of Pennsylvania (2006–2013). All research adhered to the legal requirements of the United States of America.

Results

Bioassays: Conspecific

There were dramatic differences in the behaviors directed to the conspecific and control odors in the conspecific trials (N=12). Subjects spent at least twice as much time

in proximity to the conspecific odor than to the control one (median in secs, control vs conspecific, 23 vs 48 s), and showed similar patterns in time sniffing (7 vs. 14 s) and touching (3 vs. 10 s). The differences were statistically significant for proximity ($V = 78$, $P < 0.001$, $r=0.71$, $N=24$) and sniffing ($V=5$, $P< 0.001$, $r= -0.58$, $N=24$), but not for touching ($V = 14$, $P=0.19$, $r=-0.27$). The position of odors, and which were approached first, were relatively balanced across trials (Table 2). We observed one male licking the conspecific odor, but did not observe any subjects scent-marking or placing their open mouth on the devices.

Bioassays: Kin Discrimination

Overall, subjects spent more time in proximity to, sniffing, and touching the odor from close-kin scent donors than non-kin scent donors (Table 3, Figure 3), and approached, sniffed, and touched the close-kin odor more frequently than non-kin odors (Table 3). The greatest, and statistically significant, difference was observed in time spent sniffing the stimulus tubes (Table 3). Visual inspections of the data suggest that the preference for close-kin over non-kin may be affected by which odor was first approached, but less so by the position (left/right) of the close-kin odor (Figure 4). However, the odor first approached was balanced across kin discrimination trials; subjects first approached the close-kin ($n=15$) and non-kin ($n=15$) odors equally (Table 4).

Males and females spent a similar proportion of time in proximity (Wilcoxon rank sum test: $W=110$, $n=30$, $p=0.83$, $r=-0.04$), sniffing ($W=96.5$, $n=29$, $p=0.75$, $r=-0.06$), or touching ($W=56.5$, $n=19$, $p=0.44$, $r=-0.18$) the close-kin odors. Although visual

inspection of the time subjects sniffed the odorants (Figure 5) suggests the difference in sniffing the odorants was slightly greater in males than females. Across all 46 relatedness trials we only observed an open mouth behavior once, performed by a female to a close-kin odor. Licking and scent-marking of the devices was not observed in any trials.

Microsatellite Relatedness Estimates

Overall, 81% of parent-offspring (n=61 dyads) and full siblings (n=17 dyads) were classified in a close-kin (PO or FS) category using the most likely relationship categorization, and as unrelated 8% of the time. All undefined dyads (N=7500) of genotyped individuals were classified into close-kin categories in only 10% of cases. The mean and median values of the coefficient of relatedness for parent-offspring and full-sibling dyads were similar to expected values, while they were a little higher than expected for half-sibling dyads (See Table 5, Figure 6).

The mean and median coefficient of relatedness were greater between social partners than potential partners (Table 5, Figure 6), and there was a greater proportion of close-kin MLR categorizations in the social partners than in all potential partners (Figure 7). The mean relatedness between social partners was also greater (more related) than the simulated means, and this difference was statistically significant (two-tailed $p=0.002$; Table 5, Figure 8). The differences in MLR between social partners and potential partners were also statistically significant (Chi-Square test with simulated p-value with 2000 replicates: $X^2=17.768$, $p<0.003$). Social partners that produced offspring had lower median values of the coefficient of relatedness than those that did not, but this was not

statistically significant (Table 5, Wilcoxon rank sum: W=177, p=0.65, n1=12, n2=27, r=-0.07).

There were minimal differences in the coefficient of relatedness between male-male and female-female dyads of dispersed individuals in the pool of potential partners (Table 5, Figure 6). These differences between the same-sex dyads were not statistically significant for the coefficient of relatedness (Wilcoxon rank sum: W=369960, p=0.70, N1=911, n2=804, r=-0.01), nor for MLR categories (Chi-square test: X^2=5.73, df=3, p=0.13).

Discussion

Together, our captive experiments and wild observational data are consistent with the hypothesis that owl monkeys can discriminate between individuals based on relatedness, and that cues of relatedness are used in mate choice. Wild owl monkeys showed a preference for closely related individuals in the formation of pair bonds; the mean estimate of relatedness in social partners were much higher than the simulations of random pairing. It is possible that this is facilitated by chemosignals of relatedness, as captive owl monkeys discriminate between the odor of close-kin and non-kin; males and females spent more time sniffing the subcaudal glandular secretions from unfamiliar close-kin than unfamiliar non-kin.

To the best of our knowledge, this is the first evidence of inbreeding preference in a wild non-human primate, and the first evidence of kin discrimination for unfamiliar individuals in a pair-living non-human primate. Genetic analyses of other wild non-human primate genera suggest there is active inbreeding avoidance (Huchard et al., 2013,

2017; Wikberg et al., 2017). In addition to these findings, we confirmed that owl monkeys can detect the odor of conspecifics from glandular secretions, and corroborated observational evidence that dispersal in wild *A. azarae* is not sex-biased (Fernandez-Duque, 2009).

The response displayed by captive *A. nancymaae* in our behavioral bioassays demonstrates that owl monkeys can discriminate between close-and non-kin based on pedigree. This is similar to ring-tailed lemurs (*Lemur* catta), who differentially attend to olfactory cues of individuals based on genetic relatedness and individual heterozygosity (Charpentier et al., 2010). This differs from our previous research, which did not find greater similarities in chemical distances between chemical profiles of glandular secretions between close-kin and non-kin (Spence-Aizenberg et al., accepted). There are a few potential explanations for this discrepancy. First, chemical distance may not be the best way to identify chemosignals of relatedness in the glandular secretions of owl monkeys. Second, pedigree relatedness may not be tightly correlated with chemical profiles of secretions. Finally, our previous work focused only on volatile compounds, and it is possible that chemical signals of relatedness are non-volatile. Owl monkeys do possess a vomeronasal organ (Hunter et al., 1984), suggesting that they would be able to detect non-volatile compounds.

There was also substantial variation in the degree of preference for kin, and it seems reasonable to suggest that genetic estimates of relatedness, which likely differ from those based on pedigree, might better explain variation in preference. It should also be noted that all females in this study were on contraception because we were unable to control for female reproductive phase during testing in this population, and this can affect odor

preferences of potential mates (Singh and Bronstad, 2001; Havlicek et al., 2005; Havlíicek et al., 2006; Scordato and Drea, 2007). Women on contraception show altered patterns of MHC-type preference when on oral contraception than when not (Wedekind et al., 1995; Roberts et al., 2008). Additionally, olfactory cues of relatedness are obscured by contraception in ring-tailed lemurs, and affect male preference for female odor (Crawford et al., 2011). It is unclear whether this may be the case for owl monkeys. Contraception was not found to drastically affect the volatile chemical profiles of female owl monkeys (Spence-Aizenberg et al, accepted). However, it may be that contraception may alter the odor of female glandular secretions in ways that are not captured by chemical distances, as is likely the case for relatedness, and therefore may alter the perception of the odors without showing changes in the volatile chemical profile.

The prevalence of closely related partners suggests there is some mechanism for kin discrimination in *Aotus*. The observed pattern, which differs substantially from simulations of random mating, has implications extending beyond kin recognition. Owl monkeys may have a relatively higher threshold for close inbreeding than other non-human primates, given that this is the first evidence of inbreeding preference in a non-human primate population. It seems clear that none of the mechanisms for inbreeding avoidance proposed by Pusey and Wolf (1996) are used by owl monkeys. Kin recognition may not be a mechanism for inbreeding avoidance, there is no evidence for sex-biased dispersal based on this study, nor are extra-pair copulations successful to our knowledge (Huck et al., 2014).

When considering the social and reproductive behavior of owl monkeys with respect to the three criteria that may lower the threshold for inbreeding, outlined by the Kokko

and Ots (2006) model, owl monkeys seem to fulfill all of these criteria. First, male owl monkeys invest heavily in infant care, taking on the vast majority of parental behavior, excluding nursing (Dixson and Fleming, 1981; Wright, 1984; Rotundo et al., 2005; Huck and Fernandez-Duque, 2012). Second, owl monkeys might be considered to infrequently encounter potential mates. The lack of extra-pair paternity observed in this population (Huck et al., 2014) suggest that extra-pair copulations are not a viable reproductive strategy. Owl monkeys only reproduce when they reside in an established territory. Our study area is saturated, and therefore new pairs cannot form. Thus, in order for an individual to reproduce, he/she must replace a resident after death or aggressively evict a resident (Fernandez-Duque and Huck, 2013). Replacement events are rare since the average tenure of a pair is approximately three years (Fernandez-Duque and Huck, 2013), implying that, even if groups may encounter other groups or solitary individuals frequently, there is a low encounter rate for potential mates.

Finally, it is possible that owl monkey reproduction is more similar to sequential mating than simultaneous mating. When an individual dies, he/she are replaced very quickly (Fernandez-Duque, personal communication). During these replacements, it is unclear whether there are several individuals competing for that spot, more similar to simultaneous choice, or if the nearest individual assumes a position in the group, more similar to sequential mating. If opportunities to form a pair bond are extremely limited, it may be in an individual's best interest to take any opportunity to pair and mate with an individual of the opposite sex, even if that individual is closely related.

It is also possible that those who do pair with close-kin may gain greater inclusive fitness benefits, particularly if inbreeding depression is weak. We do not yet know

whether owl monkeys experience inbreeding depression. Model predictions developed by Lehtonen & Kokko (2015) suggest that any inbreeding depression would prevent inbreeding from invading a monogamous, outbred, population. If this is correct, then the presence of close inbreeding suggests owl monkeys do not experience inbreeding depression.

Although our estimates of relatedness suggest that some of our *A. azarae* social partners were very closely related (possibly parent-offspring or full-siblings), we do not have the parentage for any of our social partners despite the population having been monitored for 21 years. Therefore, we cannot verify the pedigree relationships between the observed social partners in this study. Still, the estimates of relatedness for our known parent-offspring, full-sibling, and half-sibling dyads were fairly accurate, suggesting that they are likely to be close-kin. We also cannot verify whether they might have been familiar or unfamiliar relatives, and thus cannot rule out familiarity as a mechanism of kin discrimination.

Interestingly, there were minimal differences in estimates of relatedness between social partners that had offspring and those that did not, suggesting that relatedness did not necessarily affect whether or not a pair reproduced. However, we did not consider whether estimates of relatedness were associated with the number, survival, or reproductive success of offspring. Future work investigating this could provide us with a better understanding of the consequences of close inbreeding in *Aotus,* and whether it might cause inbreeding depression.

That owl monkeys can unequivocally detect the odor of other, unfamiliar, owl monkeys from secretions deposited on a substrate is another important finding from this

study. The ability of individuals to discriminate between, and show a preference for the conspecific over the control, odors demonstrates that a) the bioassay testing paradigm presents individuals with detectable odorants, and b) that owl monkeys can perceive odors from scent-marks deposited on a surface. This establishes that owl monkeys can use these scent-marks to communicate with conspecifics. It also opens the door to move forward with more behavioral bioassays as way to identify chemosignals in glandular secretions. Future bioassays comparing the ability to discriminate between odors based on sex or female reproductive phase (see Chapter 4), or genetic relatedness would allow us to identify whether these might be perceived by owl monkeys.

Tables

Table 1: Descriptions of behaviors recorded during the bioassay trials

Category	***Behavior***	***Description***
Device Interactions: States	approach	moves head or chest within 6 cm of a stimulus tube and remains within 6 cm for >1 second, or interacts with the stimulus tube
	sniff	nose or mouth is in contact, or within 1 cm, of stimulus tube or substrate; sniffing ends when the subject moves nose of mouth beyond 1 cm of the tube or substrate and remains distant for >1 second
	touch	makes contact with stimulus tube with one or both hands
Device Interactions: Events	lick	tongue makes contact with stimulus tube or substrate
	open mouth	mouth open and in contact with stimulus tube or substrate
	scent mark	scent marks stimulus tube with chest, face, anogenital or subcaudal gland or substrate

Table 2: Summarizing trial layout and subject performance (approaching 0, 1, or 2 odors) for 14 conspecific trials

Position of conspecific odor		Odor first approached		Odorants approached	
Left	7	Conspecific	2	Both	2
				One	-
		Control	5	Both	5
				One	-
Right	7	Conspecific	1	Both	1
				One	-
		Control	6	Both	4
				One	2

Table 3: Comparisons of behaviors directed to close-kin and non-kin odors in bioassay trials

Behavior	Odor type	Median (range)	Effect size (r)	Wilcoxon signed rank (V)	P-value
Approach (frequency)	Close-kin	4.5 (1-22)	-	-	-
	Non-kin	3.5 (1-17)			
Approach (duration)	Close-kin	24.1 s (1.5-149.3)	-0.178 (n=30)	281	0.329
	Non-kin	18.7 s (0.66-157)			
Sniff (frequency)	Close-kin	4.5 (0-20)	-	-	-
	Non-kin	3.5 (0-24)			
Sniff (duration)	Close-kin	8.6 s (0-69.2)	-0.366 (n=30)	330	0.045
	Non-kin	5.4 s (0-62.2)			
Touch (frequency)	Close-kin	1 (0-8)	-	-	-
	Non-kin	1 (0-10)			
Touch (duration)	Close-kin	2.9 s (0-29.7)	-0.196 (n=30)	188	0.284
	Non-kin	1.27 s (0-52.58)			

s: seconds

Table 4: Summarizing trial layout and subject performance (approaching 0, 1, or 2 odors) for 45 kin discrimination trials

Position of close-kin odor		Odor first approached		Odorants approached	
Left	24	Close-kin	14	Both	12
				One	2
		Non-kin	5	Both	5
				One	-
		None	5		
Right	21	Close-kin	3	Both	3
				One	-
		Non-kin	15	Both	10
				One	5
		None	3		

Table 5: Mean and median coefficient of relatedness for dyad types in the population for known relationships and all possible dyads in the pool of available mates

Dyad Type	Mean (SD)	Median (range)	N
Parent-offspring[K]	0.48 (0.17)	0.50 (0-0.86)	61
Full-sibling[K]	0.49 (0.26)	0.54 (0-0.86)	17
Half-sibling[K]	0.35 (0.19)	0.39 (0.11-0.56)	6
Social partners (MF dyads)[K,D]	0.17 (0.22)	0.05 (0-0.79)	42
with offspring [K,D]	0.17 (0.23)	0.02 (0-0.79)	27
without offspring [D]	0.18 (0.23)	0.05 (0-0.62)	12
FF-dyads*	5	0.03 (0-0.82)	804
MM-dyads*	0.11 (0.15)	0.02 (0-0.79)	911
Potential partners (all MF dyads)*	0.11 (0.16)	0.01 (0-0.86)	1751
Potential partners (simulated means)**	0.11 (0.02)	n/a (0.03-0.22)	10000

[K] known relationships verified genetically in Huck 2014, [D] relationships identified from long-term demographic monitoring of the population, * All possible dyads in the pool of available mates, **relatedness estimates for 10,000 simulated means generated for random sampling of potential mates for residents in social pairs

Figures

Figure 1: Position of two stimulus tubes were hung on the cage front 25cm apart

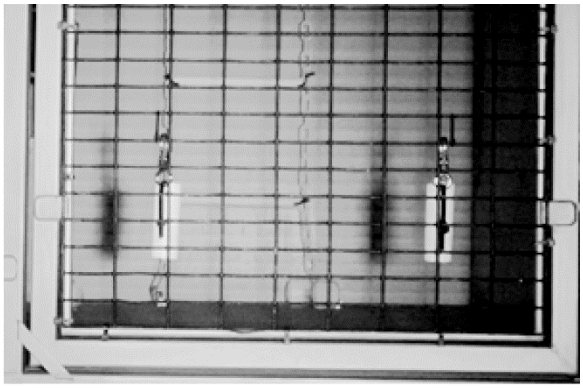


Figure 2: Example of a male sniffing and touching a stimulus tube

Figure 3: Proportion of time within a kin discrimination trial the subject spent in proximity to, sniffing, or touching the close-kin odor. Values above the 0.5 line show a preference for close-kin, and below the line for non-kin odors

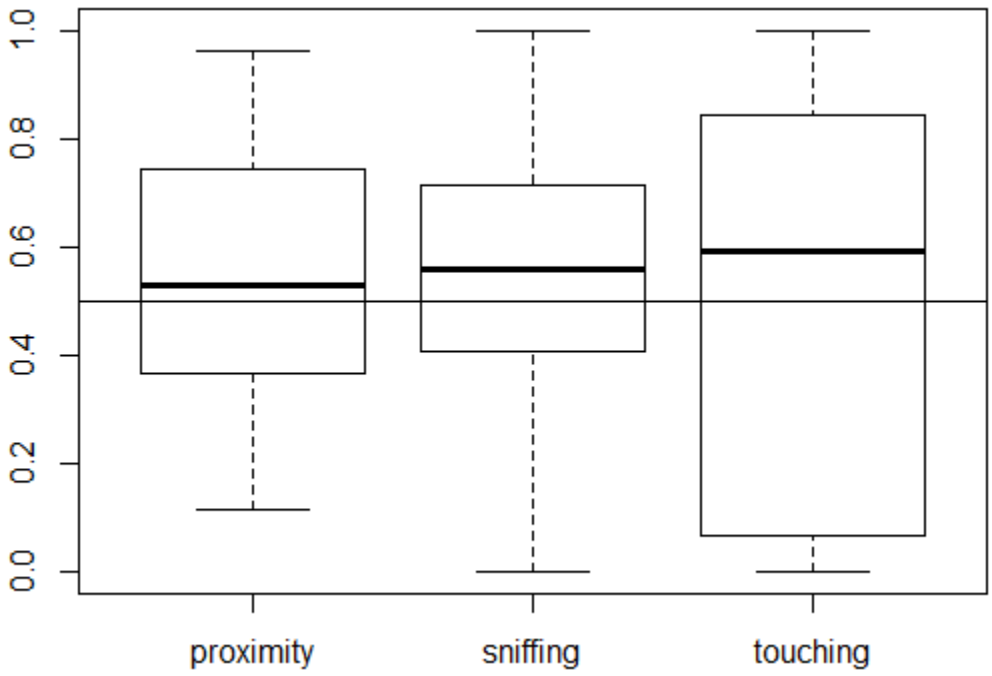


Figure 4: Proportion of time within a kin discrimination trial the subject spent sniffing close-kin odors based on the position of the close-kin odor (left/right) and the first odor approached. Preferences for close-kin (above 0.5 line) or non-kin (below 0.5 line)

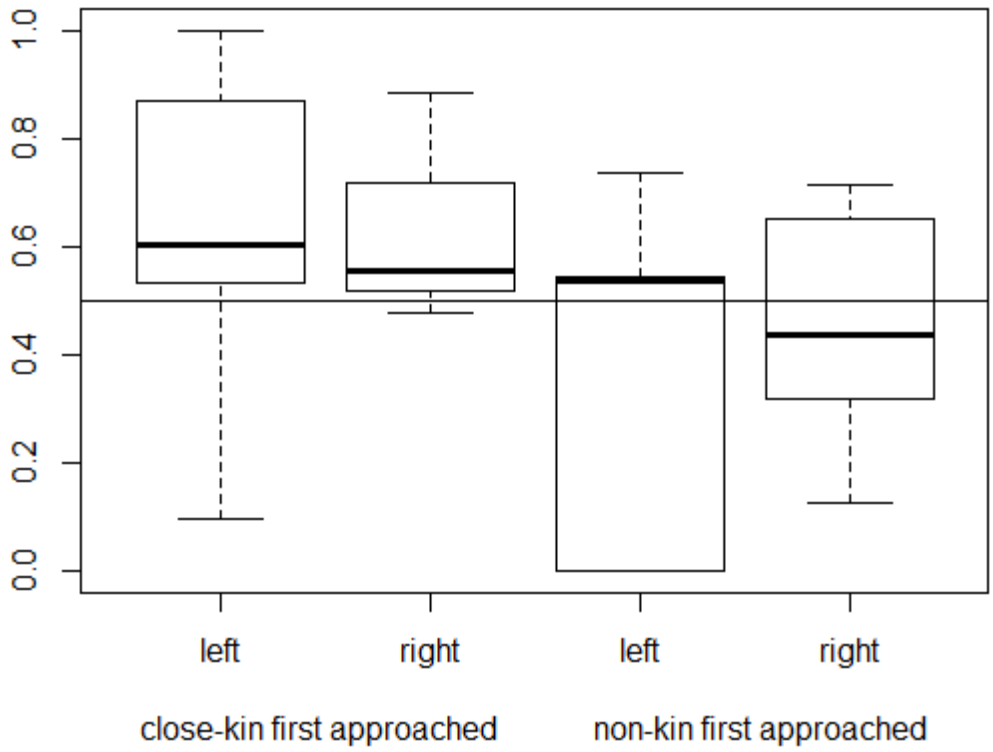


Figure 5: Mean proportion of time(s) spent sniffing close-kin and non-kin odors during kin discrimination trials by males and females. Error bars represent the standard errors of the mean

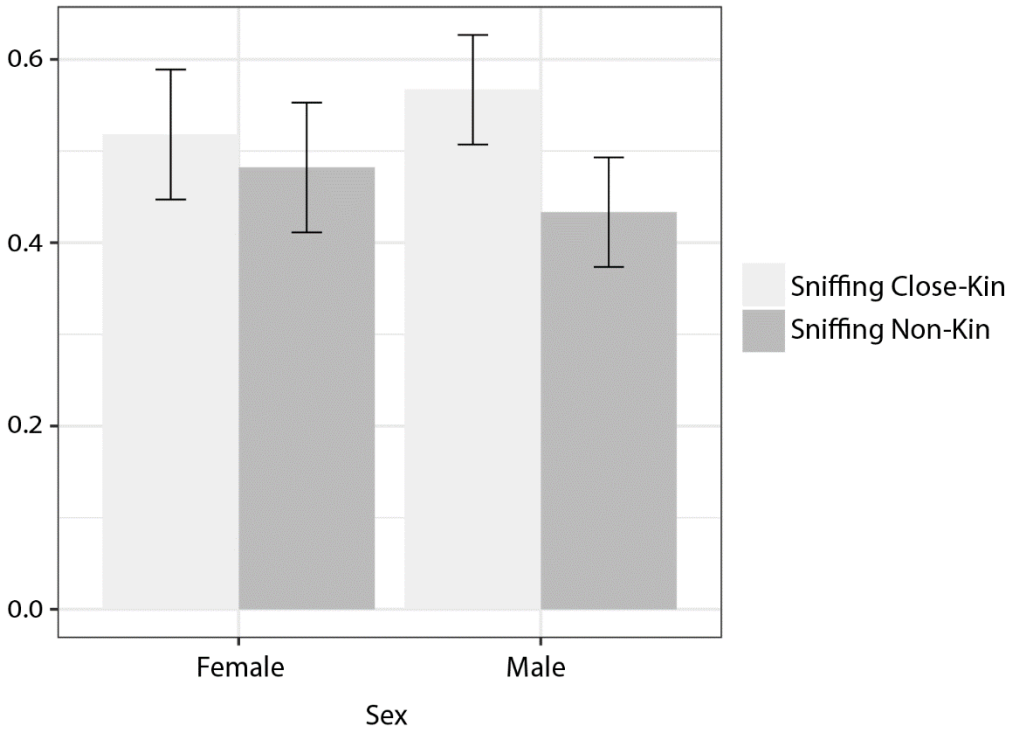


Figure 6: The estimated coefficient of relatedness generated by ML-Relate for dyads with known relationships (parent-offspring, full-sibling, half-siblings, and social partners) and for all male-female (potential partners), male-male, and female-female dyads in the pool of available mates

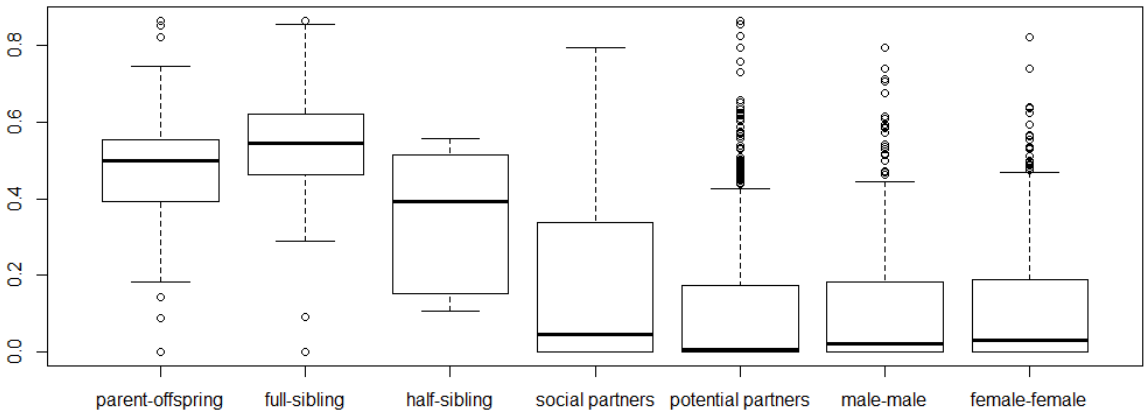


Figure 7: Most Likely Relationship (MLR) categorizations from ML-Relate for all potential partner and social partner dyads

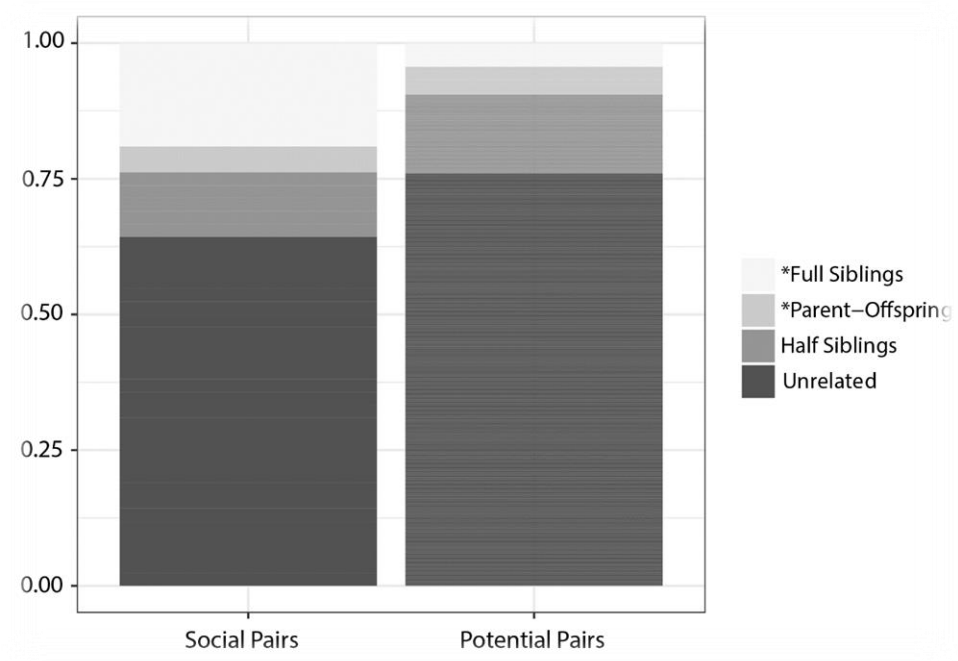
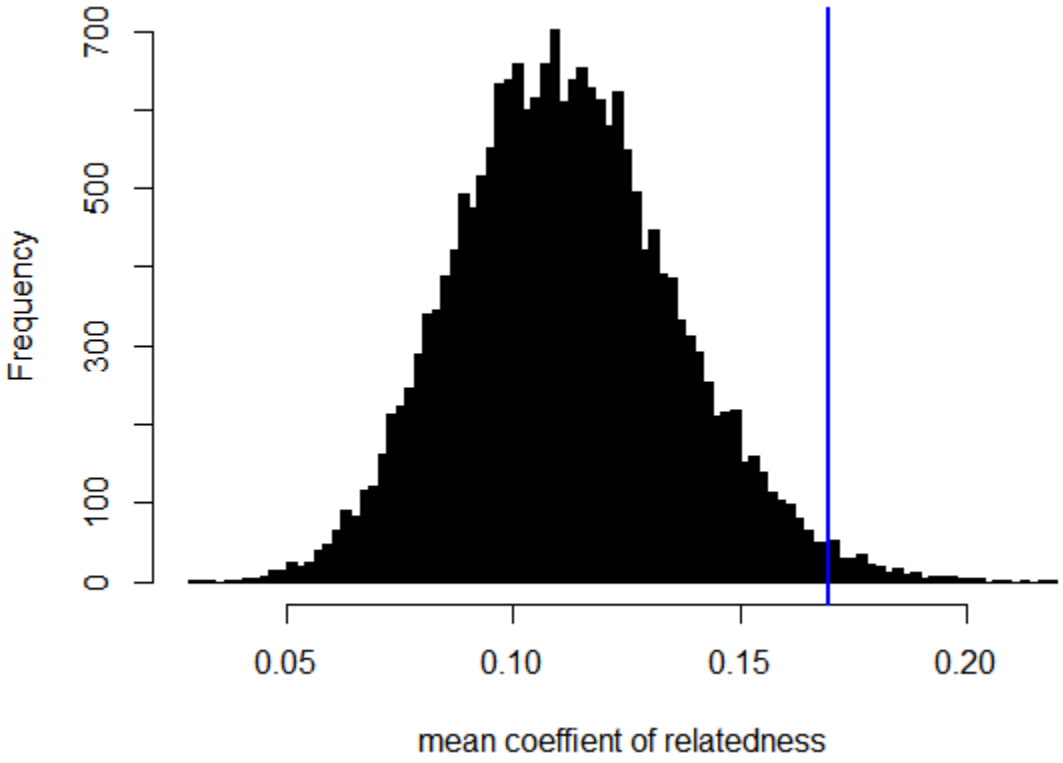


Figure 8: Distribution of simulated means (bars) when randomly sampling 42 male-female dyads from the pool of potential partners, compared to the actual mean of social partners (line)



CHAPTER 4: Can owl monkey males (*Aotus* spp.) detect ovulation of their pair mates? An experimental and observational evaluation of captive and wild pairs

Abstract

The odor of females in different reproductive states elicits differential preferences, behavior, and physiological responses across primates, including humans. Whether these odors lead to behavioral changes that ultimately improve reproductive success is less clear. Although these signals seem to be present across primates, whether these signals of fecundity exist, and how they function, in a strictly pair-living primate with little or no evidence of extra-pair paternity is unknown. Here, we coupled experimental data from a captive population of *A. nancymaae* with behavioral and hormonal observations from breeding pairs in captive *A. nancymaae* and wild *A. azarae*. We conducted behavioral bioassays (n=6) to assess whether males differentially respond to the glandular secretion of odors based on their fecundity. We also evaluated whether there is evidence that olfactory signals, or any signal of fecundity, is used within breeding pairs (n=15 captive pairs, n=11 wild pairs). We found that males can discriminate between glandular secretions based on fecundity. Males spent much more time investigating the odors of females when they were most, rather than least, fecund. However, behavioral observations from our breeding pairs did not show strong support that these signals are used between partners. Captive pairs showed only a limited increase in copulations when females were most fecund. Among wild pairs, copulations were most frequent around the time of ovulation, but they were concentrated after, rather than before, ovulation when a female is least fecund and cannot conceive. Overall, experimental evidence suggests

females emit olfactory cues of fecundity, and that unfamiliar males can detect these cues. However, these cues do not seem to be accurate enough to appropriately time sexual behavior in wild pairs.

Introduction

Concealed ovulation, or the lack of fecundity cues, was proposed as a human evolutionary adaptation co-evolving with monogamy, pair bonds, and biparental care (Morris, 1967; Alexander and Noonan, 1979; Lovejoy, 1981). We now know that concealed ovulation is not uncommon among other primates (Sillen-Tullberg and Moller, 1993), and it has been suggested that the "loss of estrus" is an evolutionary trend in the primate clade (Pawłowski, 1999). Furthermore, it has become clear that in numerous primate taxa where ovulation is visually concealed there may be chemical cues of ovulation and female reproductive status (Ziegler, 2013a; Drea, 2015). For example, exposure to the odor of cycling females elicits different responses in males depending on whether odor is sampled when the female is most, or least, fecund. Men also report higher preference for the odor of women during the follicular phase, when women are most fecund, than the odor from the luteal phase (Singh and Bronstad, 2001; Havlíček et al., 2006; Gildersleeve et al., 2012). Among non-human primates, male ring-tailed lemurs preferentially attend to the odors of females in breeding condition (Scordato and Drea, 2007), whereas males prefer peri-ovulatory females in common marmosets (Smith and Abbott, 1998) and cotton-top tamarins (Washabaugh and Snowdon, 1998). Exposure to the odor of a peri-ovulatory female results in changes in erections or sexual behavior (Ziegler et al., 1993, 2005), and elevation in testosterone levels (Ziegler et al., 2005). Although the evidence that there are chemosignals of ovulation produced by females that are detected by males is compelling, it is less clear whether these cues influence the reproductive behavior of individuals. Some observational studies suggest that behavior between sexual partners changes during times when the female is more likely to conceive

(Kendrick and Dixson, 1983; Converse et al., 1995; Carnegie et al., 2005; Thompson et al., 2011), and that chemosignals mediate these behavioral changes (Van Belle et al., 2009; Thompson et al., 2011).

As illustrated above, numerous studies have examined the presence of chemosignals of fecundity, still few have taken a comprehensive approach to investigate the proximate mechanisms and function of them. In a recent review of the state of knowledge in this area, Drea (2015) concludes that there is a dearth of studies integrating morphological, behavioral, chemical, and physiological studies in the field of primate olfactory communication, this being particularly true for research on signals of female fecundity. A more comprehensive investigation of chemosignals related to female fecundity, including chemical evidence and behavioral responses of mates, is essential to establish a solid understanding of the proximate mechanisms and function of these putative signals. For instance, there is still limited data assessing if chemosignals of fecundity result in biologically meaningful behavioral changes that lead to increases in the chances of conception, successfully mate guarding, or improving paternity certainty.

To better understand the mechanisms and function of olfactory signals throughout the primate clade, particularly in the context of female fecundity, we need to explore putative signals across various social and mating systems. As Emery Thompson & Muller (2016) discuss, the diversity in sexual behavior and its neuroendocrine regulation, and the predictability of certain features in relation to certain mating contexts, mean that we can use these features as reliable clues to understand the evolutionary history of a species. For example, if chemosignals of fecundity are in fact related to paternity certainty, sexual behavior and ovulatory signals are expected to differ in taxa with high or low levels of

extra-pair paternity. Accordingly, a study of a strictly monogamous species with low or no evidence of extra-pair paternities can provide an excellent contrast to the potential function of signals of fecundity observed in non-monogamous taxa.

We investigated the reproductive and olfactory behavior of owl monkeys (*Aotus* spp.), a pair-living, monogamous, pair-bonded primate. Owl monkeys have a relatively unique, strictly monogamous social system with low, or nonexistent, rates of extra-pair paternity (Huck et al., 2014). They show anatomical, chemical, and behavioral evidence that strongly suggest olfaction is important in within-pair communication. Anatomically, they possess an olfactory bulb that is large relative to brain size, and like other platyrrhines, they have a vomeronasal organ (Hunter et al., 1984). They also have apocrine glands throughout the body (Hanson and Montagna, 1962), and a specialized sub-caudal gland, with hypertrophic sebaceous and apocrine glands, that exhibits thicker and more densely planted stiff, specialized hairs (Hill et al., 1959; Hanson and Montagna, 1962). There is also evidence that chemical information on sex and age is encoded in sub-caudal scent gland secretions (Spence-Aizenberg et al., accepted), and that pair mates frequently engage in inspections of the partner's genitalia (Wolovich and Evans, 2007). Behaviorally, scent-marking (rubbing scent glands on a substrate), partner-marking (rubbing scent glands on their pair mate), and inspecting (sniffing the anogenital/subcaudal region of their partner) occur regularly in both captive and wild individuals (Corley et al., in prep; Wolovich and Evans, 2007; MacDonald et al., 2008).

In view of this evidence, we hypothesized that the odors from glandular secretions function as signals mediating within pair relationships; particularly with regards to the coordination of reproduction. We proposed that *Aotus* females produce a chemosignal of

fecundity, and that this signal is perceived by males. To evaluate this hypothesis, we conducted behavioral experiments to assess odor perception in captive *A. nancymaae,* and we monitored the behavior and endocrinology of breeding pairs of captive *Aotus nancymaae* and wild *Aotus azarae*. This approach allowed us to identify how putative signals are behaviorally expressed and received within breeding pairs, and whether these signals are detected through odor cues alone. We predicted that: (1) males respond differentially to female glandular secretions produced in different reproductive phases, (2) breeding pairs increase their frequency of copulation prior to ovulation when the female is most fecund, (3) female's increase marking with urine or glandular secretions prior to ovulation, (4) males increase inspections of their female partner prior to ovulation, when she is most fecund.

In addition, we also investigated the potential role olfactory communication and signatures of fecundity may play in the reproductive delay associated with newly formed pairs. Established owl monkey pairs regularly conceive offspring during the breeding season (Fernandez-Duque and Huck, 2013). However, there is a marked delay in reproduction in new pairs, who typically do not reproduce in the first breeding season and have longer inter-birth intervals than established pairs, both in the wild and in captivity (Málaga et al., 1997; Fernandez-Duque and Huck, 2013). This reproductive delay, which ultimately affects the reproductive success of the breeding pair, are rarely reported in the literature on pair-living taxa. In fact, in other taxa, delays in reproduction after forming a new pair are associated with difficulties in achieving a high quality territory (i.e. loons) (Piper et al., 2011), or with increased time traveling to find a new partner (i.e. sea horses) (Kvarnemo et al., 2000). Neither of these would explain the delay in owl monkeys, since

new pairs inherit the territory of the resident adult. We proposed that owl monkeys males must learn to identify the olfactory cues associated with ovulation in their partner, similar to the familiarity required by male macaques when using facial color to detect ovulation (Higham et al., 2011), and possibly by saki monkeys to appropriately time reproduction (Thompson et al., 2011). If the reproductive delay is mediated by chemical communication, we predicted that females in newly formed pairs will ovulate as frequently as females in established pairs, and that inspecting and sexual behaviors in newly formed pairs will not increase during the fecund phase.

Methods

Study Sites and Subjects

Our captive population of *A. nancymaae* were housed at the Owl Monkey Breeding and Research Resource (OMBRR) located in the Keeling Center for Comparative Medicine and Research (MD Anderson Cancer Center, University of Texas, Bastrop). The OMBRR houses approximately 400 owl monkeys on a semi-reversed light cycle with periods of darkness extending approximately from 1500h to 0000h. Animals were housed in one of two large colony rooms. Most individuals were housed in pairs or family groups in enclosures approximately $1.8m^3$ in size, while some individuals were housed solitarily. Water was always available to the animals, and they were fed primate biscuit with fruit or vegetable twice daily before 1500h, provided food remains available throughout the dark cycle. Enclosures were directly adjacent to one another. Groups were isolated visually from each other, and white noise (a waterfall) buffered the acoustic interactions within the rooms.

We conducted behavioral bioassays on nine solitary males in 2015. To monitor the reproductive endocrinology and behavior of breeding pairs, we studied 16 *Aotus nancymaae* male-female pairs (eight in 2013, eight in 2015). Males and females wore colored collars so that observers could positively identify individuals. None of the females were pregnant or lactating since they had been receiving contraception until the study period began. Eight of the 16 pairs were "newly-formed," with the adult male and the adult female having been introduced to each other less than a month prior to the start of data collection. The remaining eight pairs were "established" pairs, meaning two adults had resided together at least two years prior to the start of data collection.

We also studied 11 male-female pairs of *Aotus azarae* (2005: n=3, 2008: n=3, 2009; n=1, 2012: n=4) who are part of an owl monkey population ranging in gallery forests along the Pilagá and Guaycolec Rivers in Formosa, Argentina (58° 11′W, 25° 58′S). This population has been monitored regularly since 1997 as part of the Owl Monkey Project. The low levels of sexual dimorphism in *Aotus* taxa (Fernandez-Duque, 2011) make it necessary to mark individuals in order to reliably and regularly identify them. In order to do this, members of this population were darted and anesthetized using ketamine hydrochloride projected from a CO_2-powered rifle and fitted with VHF radiocollars, or ball-chain collars with colored beads, to facilitate individual identification, following established methods (Juarez et al., 2011; Fernandez-Duque et al., 2017). The resident male in one pair in 2012 died early in the study, and was subsequently replaced by a new male. All other pairs were "established" pairs who had been together more than one breeding season.

Behavioral Bioassays: Olfactory Detection of Female Fecundity

Experimental Design

We conducted a series of nine choice trials where we presented a male subject with two samples of glandular secretions collected from a female at two different times in her ovulatory cycle, following the methodology established in Chapter 2. Scent-donors and subjects were "unfamiliar" to each other, having never shared a living space, and were "non-kin," not sharing any maternal or paternal grandparents. We monitored the reproductive cycle of three female scent-donors, collected three samples from each scent-donor ten days apart, and retroactively assigned samples to the "fecund" or "non-fecund" phase based on their collection date in relation to the observed ovulatory peak based on the hormonal assays described below. We collected the samples from scent-donors by rubbing sterile cotton swabs across their subcaudal and perianal regions. After collection, we sealed the swabs in glass chromatography vials and stored at -20°C (Spence-Aizenberg et al., accepted). Prior to the start of each trial, we brought scent-donor samples to room temperature.

We presented subjects with two different odorants on "stimulus tubes," small PVC tubes approximately 5 cm in length and 2.5 cm in diameter. The tubes were reconfigured enrichment feeders, made of a material found throughout their housing, on which they frequently scent mark and inspect the marks of others. We alternated the position of the fecund and non-fecund odorants (top or bottom) so that the position of each odor type (non-kin vs. control or close-kin vs. non-kin) was balanced across trials. Immediately before the trial began, we rubbed the scent-donor swabs on the center of the stimulus

tubes, covering approximately 6.5 cm^2 (Scordato and Drea, 2007). We then placed these tubes in the subject's cage 25 cm apart (Charpentier et al., 2010).

Data Collection

The trial began when we closed the cage door after the stimulus tubes were hung, and continued for 10 minutes after the subject first approached a stimulus tube within 6cm. We digitally recorded all trials using an infrared HD Sony camera and an infrared lamp that provided additional lighting. We played back recordings of trials using Avidemux2.6 (http://www.fosshub.com/Avidemux.html). While watching the playbacks we recorded the duration of time spent sniffing each odorant. Sniffing began when the animal put its nose or mouth in contact, or within 1 cm, of the stimulus tube. Sniffing ended when the subject moved its nose or mouth further than 1cm of the tube or substrate and remains distant for >1 second. These methods for evaluating discrimination of odor were validated in Chapter 2 of this thesis.

Data Analysis

To assess whether owl monkeys can detect odors of conspecifics in the bioassay, we recorded the time that male subjects spent sniffing the fecund and non-fecund odors within trials. We compared these durations and used the Wilcoxon signed rank test to test for statistically significant differences, and "rFromWilcox" (Field et al., 2012) to calculate effect size. Female A was sampled on Day -6 (fecund) and Day 5 (non-fecund). Female B was sampled on Day 1 (non-fecund) and Day 10 (fecund) in relation to ovulatory peak 1, which corresponds to Day -13 (non-fecund) and Day -5 (fecund) in

relation to a second ovulatory peak. The third female was pregnant during both sample collections, and therefore trials in which she was the scent-donor were excluded from analysis (N=3).

Reproductive Endocrinology and Behavior of Breeding Pairs

Fecal collection and extraction

We monitored all pairs during eight consecutive weeks. In order to monitor the reproductive cycles of females within these pairs, we collected fecal samples, approximately every other day, from the adult female in each *A. nancymaae* and *A. azarae* pair. All fecal samples were collected upon evacuation. At the OMBRR, we collected feces on a tray placed under the cage, and in the field we collected feces from the leaf litter on the ground. After collection, we transferred feces to a tube filled with 5ml of a 1:1 ethanol:distilled water solution, and then we stored them in a freezer. These collection methods, validated for *A. azarae* (Fernandez-Duque et al., 2011; Corley et al., 2017) and *A. nancymaae* (Wolovich et al., 2008), show that ovulation is detectable with sample collection every two to three days. All fecal samples were shipped at ambient temperature, and then stored at -20C. We recorded wet weights at the time of collection for all *A. nancymaae* fecal samples. We recorded dry weights for *A. azarae* samples after fecal extractions were conducted, by separating all fecal material from the liquid, allowing it to dry, and weighing the dry fecal material.

Following current protocols (Corley et al., 2017), we conducted diethyl ether extractions by adding 1 ml of deionized water and 5 ml of diethyl ether to 1 ml of the liquid from the fecal sample in a culture tube. These tubes were vortexed, and the ether

layer was transferred to a second culture tube, and left to dry. The remaining sample was then re-suspended in 2 ml of phosphate buffer, and stored in duplicate at -20˚C. Most extractions (n=290) were done in the Yale Reproductive Ecology Laboratory (YREL), and some in the Penn Reproductive Ecology Laboratory (n=128). Fecal samples from *A. azarae* collected in 2005, 2008, and 2009 had been previously assayed and the resulting data published (Fernandez-Duque et al., 2011).

Hormone Assays

We used DetectX Immunoassay kits from Arbor Assays (Ann Arbor, MI) to estimate levels of estrone-3-glucuronide (E1G, a secreted estradiol) and pregnanediol-3a-glucuronide (PDG, a metabolite of progesterone). E1G and PDG have successfully identified ovarian cycles in owl monkeys (Fernandez-Duque et al., 2011; Corley et al., 2017). We conducted all assays in the YREL following the Arbor Assays protocol. The E1G and PDG DetectX Immunoassay kits were previously validated for *A. azarae* (Corley et al., 2017), and were validated for *A. nancymaae* using parallelism and accuracy by creating serial dilutions of pooled samples. The pooled sample dilutions fell directly on the standard curve.

Prior to running the assay, we allowed samples to come to room temperature, and diluted them with Arbor Assay buffer as needed. Dilutions for the samples were made as follows: 1:90 (*A. nancymaae* E1G), 1:40 (*A. azarae* E1G), 1:20 (*A. nancymaae* PDG), 1:10 (*A. azarae* PDG). Any samples that exceeded the threshold, or had too much variation in the duplicate samples, were rerun at an adjusted dilution. Mean inter-assay coefficients of variation (CVs) were 8.9% for E1G (9.2% = high control; 8.5% = low

control) and 9.1% for PDG (9.2% = high control; 8.9% = low control). The mean intra-assay CVs were 11.7% for E1G and 11.4 for PdG. Values of E1G are reported as ng/g wet feces, and PDG as ug/g wet feces for *A. nancymaae*, and as ng/g dry feces, and PDG as ug/g dry feces for *A. azarae.*

Female ovulatory cycles

For identifying ovulatory peaks, we used the criterion of an increase in PDG levels greater than two standard deviations above the mean follicular level (Corley et al., 2017), and the visual inspection of the hormonal profiles of each individual female. Since fecal PDG in some platyrrhines typically lags 0-2 days (Ziegler et al., 1996, 1997; Campbell et al., 2001), we presumed that ovulation occurred prior to the rise in PDG, and estimated the day of ovulation (Day 0) as one day prior to sample collection. Based on established knowledge of platrryhine reproduction (Ziegler et al., 2009b), we considered the fecund phase (the follicular phase) to precede and include the day of ovulation, and considered the non-fecund phase (the luteal phase) to follow ovulation.

Aotus have a follicular phase that is approximately six days long, and a luteal phase that lasts approximately ten days (Bonney et al., 1980). Because our lag time was estimated, we conservatively considered the day of ovulation (Day 0) and five days prior (Days -1 through -5) as the fecund phase, likely encompassing most of the follicular phase. We considered the eight days following ovulation (Days 1 through 8) as the non-fecund phase, which encompassed much of the luteal phase, or gestation in cases when the cycle was conceptive.

In cases where females had more than one consecutive ovulatory cycle, we estimated cycle length by calculating the number of days between E1G nadir points and between peaks, and calculated cycle length following the criteria of Corley et al. (2017), in which we did not consider cycles exceeding 25 days to be consecutive cycles. Based on estimates from another study reporting a length of ~117 days in captive *A. nancymaae* and 121 days in captive *A. azarae* (Wolovich et al., 2008), and 120 to 126 days in wild *A. azarae* (Fernandez-Duque et al., 2011), we identified conceptive cycles by counting back ~120 days from parturition, and identifying the nearest ovulatory peak that was followed by sustained high levels of E1G and PDG, which indicated gestation. Gestation length was calculated by subtracting the estimated ovulation date of the conceptive cycle from parturition. For each female we calculated minimum, maximum and mean values of E1G and PDG excluding conceptive cycles and gestation. For females with conceptive cycles, we report the peak values of PDG and E1G. We also report the number of ovulatory peaks and conceptions in new and established pairs. We also used eight ovulatory peaks that were identified in a previous study in seven female *A. azarae* during 2005, 2008, and 2009 (Fernandez-Duque et al., 2011).

Behavioral data collection

We collected behavioral data from *A. nancymaae* and *A. azarae* individuals during 20-minute focal periods following sampling procedures and ethogram as detailed in the Monogamous Primate Project protocols (Spence-Aizenberg et al., 2016), and, for our observations of captive individuals, modified to focal-dyad sampling to record simultaneously the behavior of both the male and the female (Wolovich and Evans,

2007). During focal data collection, we used all-occurrence sampling of social, sexual, and olfactory behaviors of interest (Table 1) that involved the focal animal; some behaviors were recorded only in captive animals. Data were recorded using a digital recorder, transcribed, and transferred to a database. During 2012, all copulations in wild pairs that were observed outside of focal sampling were recorded ad libitum. All observers were trained by experienced researchers.

We collected 731 focal samples of behavioral data on the captive pairs (median: 46, range: 29-62), representing 244 hs of observations from new (115 hs) and established (130 hs) pairs. We collected 258 focal samples (86 hs) during the fecund (114 focals) and non-fecund (144 focals) phases (Table 2).

We collected 334 focal samples from the wild *pairs* (median 35, range: 19-50), representing 111 hs of behavioral data. Forty-one hours (123 focals) of behavioral observations occurred in the fecund (34 focals) or non-fecund (89 focals) phase (Table 2).

Behavioral Data Analysis

For analyses of fecundity, we only used focal samples collected during the fecund (Day -5 through Day 0) and non-fecund phase (Day 1 through Day 8, Table 2). To compare behavior between newly-formed and established captive pairs, we used all focal samples except those collected during gestation. We calculated frequencies of social, sexual, and olfactory behaviors for each pair as hourly rates, by averaging the number of times each behavior was observed across all focal samples in each phase (fecund and non-fecund), then multiplying by three to compute the average number of times each

behavior was observed per hour. Using these averages, we compared median hourly rates across individuals in the fecund and non-fecund phase, and in new and established pairs.

Additionally, we built generalized linear mixed models with the captive owl monkey data to examine: 1) the potential relationships between the behaviors of pair mates and fecundity, 2) whether new and established pairs differ in behavioral patterns during the two phases, and 3) if new pairs showed relatively fewer changes in connection with the two different phases. Because ovulatory peaks were identified after data collection, we could not balance focal collection equally across phases, pairs, or observers. Therefore, we assigned pair ID and observer ID as random effects in all models to account for unequal contributions of subjects and observers. We developed four sets of models, for 1) copulations (mounting), 2) female marking behavior (urine washing, scent-marking with subcaudal, pectoral, and face), 3) male investigations (anogenital sniffing), and 4) female proceptive behavior (female approaches and presents). We included ovarian phase (fecund/non-fecund) and pair type (new/established) as fixed effects. We used an information theoretical approach (Burnham and Anderson, 2002) to compare a set of candidate models including the following set of fixed variables: 1) ovarian phase (non-fecund or fecund); 2) an interaction between ovarian phase and pair type (established or new pairs); and 3) a null model to ensure the candidate models are appropriate (Dochtermann and Jenkins, 2011). We calculated the Akaike Information Criterion corrected for small sample sizes (AICc) to compare models, and the AICc weights and evidence ratio to evaluate the probability of each model (Burnham et al., 2011). We report the model output for the model with the lowest AICc, and the cumulative AICc weight for all parameters. We fit models with 'lme4' (Bates et al., 2012) and a Poisson

distribution with the Laplace approximation (Bolker et al., 2009), except the female proceptivity models, which were fit with a negative binomial using the 'glmmadmb' (Skaug et al., 2011). We selected the best models using the package 'AICcmodavg' (Mazerolle, 2013). None of the models were overdispersed, satisfying assumptions of the Poisson distribution.

We compared behavioral frequencies of new and established pairs using all focals, excluding gestation, because focal sample collection from them was relatively well-balanced across most observers in the captive pair (Table 3). We statistically compared these groups using the non-parametric Wilcoxon Rank Sum test.

Results

Olfactory Detection of Female Fecundity

The fecund samples were the preferred odorant in all six trials. Each male spent, on average, 3.5 times sniffing the fecund odorant over the non-fecund odorant (mean time sniffing: pref: 49 s, nonpref 14 s). This difference was statistically significant ($V=78$, $n1=6$, $n2=6$, $p<0.05$, $r=-1.0$).

Reproductive Endocrinology of Females

Aotus nancymaae

We identified 23 ovulatory peaks in 15 captive females. One female had three peaks, six had two, and eight had only one. We could not definitively define ovulatory peaks for the 16th female. Cycle length, estimated by E1G nadirs, ranged between 13 and 32 days (median: 22 days, N=8 females) and estimated by E1G peaks ranged between 18 and 36

days (median: 25 days, N=8 females). When removing cycles longer than 25 days, cycle length, as estimated by E1G nadirs, ranged between 13 and 20 days (median: 17 days, N=5 females), and as estimated by E1G peaks between 18 and 24 days (median: 21 days, N=5 females).

Five females conceived, with three conceiving more than two weeks prior to the end of the study. Four of the five pregnant females conceived on their first detected ovarian cycle. Gestation length ranged between 117 and 140 days (median: 123 days, N=5). Mean E1G and PDG values were at least twice as great during gestation than average cycling values.

During routine animal handling, we observed two females with several drops of vaginal blood five and eight days before the estimated ovulation dates of their conceptive cycles.

Aotus azarae

We identified eight ovulatory peaks in four wild females monitored during 2012. Two females had three peaks, and two had two peaks. Cycle length estimated by E1G nadirs ranged from 13 to 25 days (median=18, n=3 females) and by E1G peaks ranged from 19 to 48 days (median=28, n=4 females). When excluding cycles longer than 25 days, cycle length estimated by E1G nadirs did not change, but estimated from E1G peaks ranged from 12 to 23 days (median=18, n=3 females). None of these females conceived. As with the captive females, there was a wide range of variation in E1G and PDG levels across females (Table 4).

Sexual Behavior of Breeding Pairs

Aotus nancymaae

We observed 83 copulations over the course of our study in 15 of the 16 captive pairs. Pairs copulated between 1 and 16 times (median: 4 copulations). Of the 83 total copulations, 28 were observed during the fecund or non-fecund phases, and three occurred after conception. The remaining 52 occurred during times that we were unable to classify as either a fecund or a non-fecund phase because they did not directly precede or follow an identified ovulatory peak. The duration of the 70 timed copulations varied between established and new partners. The former exhibited shorter copulations (median: 20s, range: 3-81s) than the latter (median: 31s, range: 4-130s; Wilcoxon rank sum test: 25, $p=0.63$, $r=-0.13$, $N_1=6$, $N_2=7$). In addition to the 83 copulations, we observed seven "copulations" that were positioned away from the genitals. In these instances, males mounted the female and thrusted while the anogenital region was near his partner's head, arm, or side. These "copulation" events were performed by three males in newly formed pairs, and are excluded from all statistical analyses.

Aotus azarae

We observed 20 copulations in the wild *A. azarae* pairs. Four copulations were observed during focal sampling between 2005 and 2009, and six were observed during focal sampling during 2012. Ten additional copulations were observed and recorded ad libitum during 2012. Twelve of the 20 copulations occurred during the fecund (n=3 copulations) or non-fecund (n=9 copulations) phase. Additionally, 11 of the 16

copulations observed during 2012 were between the adults in a newly established pair that formed during the study.

Fecundity, Sexual Behavior, and Olfactory Behaviors in Breeding Pairs

Aotus nancymaae

There was limited support for our hypothesis that copulations would increase during the fecund phase. The probability of the best model, including only ovarian phase as a fixed effect, was only 46%, and only 1.2 times more likely than the null model. The model output indicated that during the fecund phase, a pair would engage in about 1.8 copulations more (0.6 per focal) per hour than in the non-fecund phase (Table 5). Still, the cumulative AICc weight for the ovarian phase parameter was not high. There was no support for an interaction between phase and pair type. Similar differences of copulation frequency based on fecundity phase were also evident when looking at mean frequencies per pair. On average, there was a tendency for pairs to copulate more frequently in the fecund phase than the non-fecund phase (Table 6, Figure 1).

The best model for female marking behaviors showed an interaction between ovarian phase and group type (Table 7). Ovarian phase has a stronger positive relationship to marking behavior in new pairs than established pairs, so that new pairs mark more frequently in the non-fecund phase than the fecund phase, with a rate of approximately three more scent-marks per hour. This model is 4.5 times more likely than the null model.

There was no support to suggest males altered their investigative behavior across the ovarian cycle. The model including an interaction between ovarian phase and group type was nearly equally likely as the null model, with only a 36% probability (Table 8). When

looking at average values per pair, we found the median values and the range were actually much greater during the non-fecund than the fecund phase (Figure 2).

There was also no evidence to suggest that females engaged in proceptive behavior more frequently during the fecund phase. In fact, the null model had the lowest AICc, and was 1.2 times more likely than the model including ovarian phase (Table 9).

Regarding other social and sexual behaviors, males more frequently performed partner-marking and arching in the non-fecund than the fecund phase (Table 6, Figure 3, Figure 4). Overall, males seemed to scent-mark more frequently than females in both the fecund and non-fecund phase (Table 6). All other social, sexual, and olfactory behaviors show no marked differences in frequency between the non-fecund and the fecund phase (Table 6).

Aotus azarae

Among the *A. azarae* pairs, copulations were most frequently observed in the fecund and non-fecund phase than periods that were undefined. However, contrary to our predictions, the majority of these copulations took place in the early part of the non-fecund phase (Table 6, Figure 5). It is important to note that pairs were not as well sampled during the fecund phases as the non-fecund phases (Table 2), and this pattern may be a byproduct of sampling bias. There were no consistent patterns in other investigative or marking behaviors observed in the *A. azarae.*

Differences between New and Established Pairs

Among the *A. nancymaae,* in the rates of copulation observed between new and established pairs were similar (Table 6, Figure 1). However, this was not the case in the *A. azarae* pairs. Although we only observed the formation of one new pair in our study, this pair was responsible for more than half of the copulations.

In captivity, we observed a similar number of ovulatory peaks in females from new (n=12 peaks) and established (n=11 peaks) pairs. Three of eight females in established pairs, and two in newly formed pairs, conceived.

Some behaviors did differ noticeably based on the duration of the pair. There was a strong trend for females in new pairs to sniff their male partners more frequently than females in established pairs (Table 10, Figure 6). There was also a tendency for greater genital sniffing by males and females in established pairs (Figure 7; Table 10), and for males to partner mark more frequently in established pairs (Figure 3; Table 10). Males and females in new pairs arched more frequently than individuals in established pairs (Figure 4; Table 10). None of these differences were statistically significant (Table 10). All other social behaviors were observed with similar frequency in both new and established pairs.

Discussion

We found compelling evidence that owl monkeys can detect differences in fecundity of unfamiliar females in behavioral bioassays. This finding suggests that, using chemical cues from the subcaudal and perianal secretions of females, males can discriminate between a more and less fecund sample. That males spend substantially more time

investigating the secretion collected when the female was more fecund suggests they are more interested in this odor. This ability to discriminate odors based on reproductive state parallels observations other non-human primates (Smith and Abbott, 1998; Washabaugh and Snowdon, 1998; Scordato and Drea, 2007) and humans (Singh and Bronstad, 2001; Havlíček et al., 2006; Gildersleeve et al., 2012).

The relationship between copulations and fecundity differed depending on the context. Our captive pairs showed very frequent copulations that occurred with similar frequencies throughout the study regardless of female fecundity, whereas wild pairs showed a much more defined relationship between fecundity and sexual behavior. Overall, among captive breeding pairs there was a slightly greater chance that a pair would copulate during the fecund phase than the non-fecund phase, but this model was not much more likely than the null model, suggesting ovarian phase does not play an integral role in copulatory behavior.

Among the wild pairs, however, the frequency of copulations within the fecund or the non-fecund phase was greater than during periods of time that we could not define. Interestingly, this increase was mostly due to copulations occurring in the non-fecund phase, after ovulation would have occurred. In part, the greater concentration of copulations in the non-fecund phase might be a by-product of unequal sampling across the phases, which was approximately three times greater during the non-fecund phase. Some observations of wild primate populations have found copulations increase during the peri-ovulatory period, defined as three days before and after the observed hormonal peak indicative of ovulation (Carnegie et al., 2005; Van Belle et al., 2009). If this study had followed this criterion, we might find a similar pattern as many of the copulations

among wild *A. azarae* occurred 1-3 days after the estimated ovulation date. This pattern of imprecise timing of mating behavior suggests that there may be a signal of ovulation, but one that is broad enough to persist beyond the point where conception is possible in wild owl monkeys, and perhaps other New World primates, as well.

Females in captive pairs increased their frequency of marking during the fecund phase, but there was also an interaction between the duration of the pair and female marking. Females in new pairs showed more frequency marking in the non-fecund phase than those in established pairs. This difference suggests that females in new pairs may not use their marking behavior to advertise fecundity as accurately as females in established pairs. There was no clear evidence to suggest that females modify their advertisement of scent based on fecundity. Similarly, there was no evidence for proceptivity in female behavior during the fecund period, as the null model best explained captive female proceptive behavior. There were no significant differences in presentation to males by captive females, or approaching males in captive and wild pairs. Other behaviors that might be considered proceptive, such as grooming or food sharing, also did not show any change based on ovarian phase. This observation suggests that any increase in copulations were not related to females actively seeking copulations with males.

We observed more genital inspections in the non-fecund than the fecund phase in both captive and wild owl monkey pairs. Still, within captive pairs, we found the model including an interaction between ovarian phase and group type was nearly equivalent to the null model. When looking at behavior over our entire data set, captive males performed genital inspections of their partner frequently throughout the study. Hourly rates were greater during the overall study than they were during the fecund or non-

fecund phase. Similarly, saki monkeys also engage in genital inspections regardless of female reproductive state (Thompson et al., 2011). It is possible that constant monitoring may be sufficient to identify approximate times of fecundity when in a monogamous pair, whereas in non-monogamous taxa, males may have to intensify inspections to monitor fecundity and actively mate guard against other males within the group, such as been observed in howler monkeys (Van Belle et al., 2009).

The previously observed delay in reproduction in newly formed pairs (Fernandez-Duque and Huck, 2013) does not seem to be caused by a lack of ovulations in recently formed pairs. In captivity, the newly formed pairs were observed to cycle and conceive at similar rates to females in established pairs. In the wild pair, the female whose partner was replaced during the study also continued cycling. Finally, we had proposed that males may require time to learn an individual female's signals of ovulation, similar to the learning required by male macaques to recognize facial changes related to ovulation in females (Higham et al., 2011). Our data do not support this idea. With the exception of female marking, olfactory behaviors between new and established pairs did not differ based on female fecundity in captive *A. nancymaae*. This fact, coupled with the overwhelmingly strong response by males toward the odor of unfamiliar fecund females suggests that familiarity is not required detect chemosignals of ovulation in owl monkeys, and the reproductive delay is not mediated by chemical communication.

The behaviors between mates that showed the greatest differences between new and established captive pairs was female sniffing, with females in new pairs sniffing their partner more frequently than in established pairs. Also, males tended to groom more in new pairs, whereas females tended to perform genital inspections more frequently in

established pairs. The similar frequencies in copulations between new and established pairs contrasts with the difference observed in a different population of captive *A. nancymaae* (Wolovich and Evans, 2007). Other behavioral differences reported by Wolovich and Evans (2007) were also not found in this study, including females in new pairs more frequently scent-marking and performing genital inspections. In fact, we found that females in established pairs engaged in more genital inspections of their partner than those in new pairs. Like Wolovich and Evans (2007), we also did not observe any marked differences in male behavior between males in new and established groups, although we did observe a tendency for males in new groups to groom more frequently. Interestingly, although we only observed one newly formed pair in the wild *A. azarae*, this pair was observed to copulate much more frequently than established pairs. It is unclear if this relationship would hold with a larger sample size.

Overall, the sexual behavior in the wild *A. azarae* pairs showed much stronger evidence that breeding pairs may use some signal of fecundity to coordinate reproductive efforts. This pattern might be muted in captivity given the much greater frequency of sexual behaviors observed. In wild populations, time and energy devoted to social and sexual behavior may be limited by time spent foraging and traveling. Alternatively, we know that olfaction is an essential component of pair bonding behavior in socially monogamous prairie voles (*Microtus ochrogaster*), as the removal of the vomeronasal organ or the olfactory bulb diminishes the development of partner preference in pair bonded voles (Williams et al., 1992; Curtis et al. 2001). If odors function to facilitate bonding in owl monkeys as well, then perhaps odor, and olfactory behaviors, play a

greater role in forming and/or maintaining that bond, rather than signaling female fecundity.

The observations of vaginal bleeding have been reported in owl monkeys following a spontaneous abortion (Schuler et al., 2007). It is possible that this is also the case in one female, although our monitoring of her did not precede the vaginal bleeding long enough to determine whether she might have been pregnant. The second female was unlikely to be pregnant. Her E1G and PDG levels were extremely low and virtually undetectable until after the vaginal bleeding was observed. Interestingly, vaginal bleeding in howler monkeys coincides with basal hormonal levels, but is only visible through vaginal cytology (Kugelmeier, 2011).

The bioassays provide strong evidence for chemosignals of female fecundity, but future studies incorporating more scent-donors and trial subjects would strengthen this evidence. The observations from wild A. *azarae* suggest that these signals may not be fine-tuned, but that pairs do concentrate reproductive efforts around ovulation. Together, the data suggest that observations of interactions between pairmates from a wild population may provide more biologically meaningful information than observations from captive groups. However, the use of captive individuals in the bioassays is critical for identifying chemical cues as a potential source for a signal of ovulation. Finally, the frequency with which olfactory behaviors were observed outside of reproductive periods suggests that they serve additional purposes within male-female relationships apart from coordinating reproduction.

Tables

Table 1: Ethogram of behaviors observed and recorded

Behavior	***Definition***
Copulations	The male mounts the female, while moving his pelvis repeatedly
Genital inspections	Sniffing, licking, or exploring the anogenital area, or urine, of the partner
Presents	The female places body for mating, grooming, touching, or inspection, typically opening her arms and/or exposing her abdomen
Partner marking	The subcaudal and/or anogenital area is rubbed on another individual, typically across their back
Sniffing	Places nose/mouth <1 cm to their partner's body, excluding the anogenital area, but is not grooming
Subcaudal marking	The subcaudal region is in contact with a substrate and the body is slid forward or laterally moving the rear part of the body
Pectoral marking	The chest region is moved with pressure and friction against the substrate by sliding the body forward. It may also be pressed in a downward motion with hands and/or arms
Face marking	The face is in contact with a substrate and the cheek is slid forward or laterally against the substrate
Urine washing	Hands are wet with animals own urine and then rubbed on some part of its body
Arching	To raise up on feet, or feet and hands, while raising the back and sometimes bouncing
Approaches	Moves to within body length (in captivity) or 0.5 m (in wild) of a stationary individual and stays for at least 3 sec
Grooming	Uses the hands or mouth to manipulate the hair of another individual with gaze directed at the part of the body being manipulated
Food Sharing	Feeding from the same piece of food another individual is feeding from, without animosity from either
Touching	Place hand(s) on another individual, but is not grooming
Aggression	Grabbing, hitting or biting another individual. It can include vigorous grasping, pulling or slapping at another, and may occur together with biting
Nose to nose	Individuals bring their noses within a few centimeters of one another, sometimes even touching

Table 2: Number of focal samples collected from each pair during each fecundity phase (fecund or non-fecund) in captive *A. nancymaae* pairs and wild *A. azarae* pairs

***A. nancymaae* pair**	**Pair type**	**Fecund**	**Non-fecund**	***A. azarae* pair**	**Pair type**	**Fecund**	**Non-fecund**
Ailyn	E	6	7	C0-2005	E	0	18
Amber	N	8	7	C0-2008	E	10	8
Appa	N	6	5	C0-2012	E	1	9
Aunt Beru	E	7	14	CC-2009	E	6	6
Cal	E	6	6	D100-2005	E	4	4
Charlette	E	6	9	D100-2008	E	0	0
Cherry Blossom	N	5	5	D500-2012	E	1	2
Ione	N	3	5	D800-2012	E	3	5
Lillian	E	10	11	E500-2005	E	5	10
Noel	N	12	17	E500-2008	E	0	0
Olivia	N	8	15	E500-2012	N	4	27
Princess Leia	E	10	10				
Samara	E	7	9				
Syrah	E	4	5				
Tamarin	N	16	19				

E: established pair; N: newly-formed pair

Table 3: Number of focal samples collected by observers from established and new pairs collected in captive *A. nancymaae* pairs (n=15)

	Established	New
Observer 1	134	115
Observer 2	96	91
Observer 3	93	94
Observer 4	35	32
Observer 5	29	12

Table 4: Hormone values for E1G (ng/g wet feces) and PDG (ug/g wet feces) for captive *A. nancymaae* females and EIG (ng/g dry feces) and PDG (ug/g dry feces) for wild *A. azarae* females

			Non-conceptive cycles		Conceptive cycles	
	Individual	**Identified ovulatory cycles (N)**	**E1G mean *(range)***	**PDG mean *(range)***	**E1G max**	**PDG max**
A. nancymaae	Ailyn	1	3037 *(1618-5309)*	22 *(11-33)*	7538	58
	Amber	1	2458 *(1233-4499)*	12 *(6-23)*	3200	41
	Appa	2	2579 *(258-9210)*	26 *(2-83)*		
	Aunt Beru	2	4908 *(207-28548)*	57 *(1-233)*		
	Cal	2	1526 *(266-4844)*	12 *(1-37)*		
	Charlette	1	4763 *(362-13592)*	35 *(0-236)*		
	Cherry Blossom	1	3702 *(320-10350)*	25 *(4-79)*		
	Ione	1	1212 *(280-2092)*	9 *(1-17)*	3516	38
	Lillian	2	2141 *(291-6818)*	13 *(0-56)*		
	Noel	2	3566 *(945-11840)*	28 *(5-76)*		
	Olivia	2	2296 *(110-7792)*	8 *(0-20)*		
	Orange Blossom	n/a	4787 *(469-13905)*	27 *(1-72)*		
	Princess Leia	1	1039 *(110-3670)*	1 *(0-7)*	9999	10
	Samara	1	1507 *(11-7718)*	15 *(0-108)*		
	Syrah	1	3209 *(4-15535)*	27 *(0-104)*	21140	427
	Tamarin	3	4283 *(3.6-16941)*	25 *(0-147)*		
A. azarae	E500 female	3	2171 (*80-8054*)	37 (*0.3-184*)		
	Celina	3	960 (*133-5745*)	15 (*0.5-35*)		
	Doly	1	2445 (*268-7384*)	47 (*4-182*)		
	Divertida	1	3423 (*178-12587*)	54 (*0.3-145*)		

Table 5: Model comparisons for the frequency of copulations in captive *A. nancymaae* pairs, and the output from the model with the lowest AICc

Model Comparison			
Fixed Effects	*Delta AICc*	*AICc Wt*	*K*
Phase	0	0.46	4
Null	0.36	0.38	3
Phase * Group Type	2.17	0.16	6

"Best" model output		
Random effects	*Variance*	*SD*
Pair ID	0.267	0.52
Observer ID	0	0

	Estimate	*SE*	*z*	*Pr(>|z|)*	*Cumulative AICc weight*	*N models including variable*
Intercept	-2.17	0.34	-6.45	<0.001		
Phase	-0.63	0.41	-1.54	0.124	0.62	2

SE: standard error; SD: standard deviation

Table 6: Median (range) hourly rates for olfactory and sexual behaviors in the fecund and non-fecund phases in 15 *A. nancymaae* pairs and 11 *A. azarae* pairs

		***A. nancymaae* ovarian phase**		***A. azarae* ovarian phase**	
	Sex	***Non-fecund***	***Fecund***	***Non-fecund***	***Fecund***
Copulations		0 (*0-1.2*)	0.38 (*0-1.3*)	0 *(0-0.2)*	0 *(0-0.5)*
Genital inspections	Male:	0.63 (*0-7.80*)	0.38 (*0-3.60*)	0 *(0-0.4)*	0 *(0-0)*
	Female:	0.2 *(0-2.1)*	0 *(0-2.1)*	0 *(0-0)*	0 *(0-0)*
Presents	Female:	0 (*0-0.86*)	0 (*0-0.38*)		
Partner marking	Male:	0 (*0-5.4*)	0 (*0-2.4*)		
	Female:	0 (0-9)	0 (0-4.5)		
Sniffing	Male:	3.6 (*0-24*)	3 (*0-28.20*)		
	Female:	2.14 (*0-4.8*)	2.43 (*0-6*)		
Subcaudal marking	Male:	0.33 (*0-14.1*)	0.43 (*0-11.4*)	0.6 *(0-3)*	0 *(0-2.4)*
	Female:	0.21 (*0-10.2*)	0 (*0-12.50*)	0.3 *(0-11.5)*	0.7 *(0-3)*
Pectoral marking	Male:	0 (*0-0*)	0 (*0-0.38*)p		
	Female:	0 (*0-0*)	0 (*0-1*)		
Face marking	Male:	0 (*0-1.67*)	0 (*0-1*)		
	Female:	0.20 (*0-1.8*)	0.30 (*0-1.8*)		
Urine washing	Male:	0 (*0-1.2*)	0 (*0-1*)	0.5 *(0-3)*	0.6 *(0-2)*
	Female:	0 (*0-1.8*)	0 (*0-2.25*)	0 *(0-1.5)*	0.2 *(0-1)*
Arching	Male:	0 (*0-1.8*)	0 (*0-0.86*)		
	Female:	0 (*0-2.4*)	0 (*0-1.8*)		
Approaches	Male:	20.4 *(0-53)*	18 *(2.3-46.7)*		
	Female:	13.2 *(1.8-37.4)*	14.5 *(1.5-42.0)*	0 *(0-1)*	0 *(0-1.5)*
Grooming	Male:	0.3 *(0-4.0)*	0.3 *(0-1.3)*		
	Female:	0 *(0-3.0)*	0 *(0-1.2)*		
Food Sharing	Male:	0 *(0-1.3)*	0.2 *(0-1.0)*		
	Female:	0.3 *(0-1.3)*	0.4 *(0-1.5)*		
Touching	Male:	2 *(0-9.3)*	1.5 *(0-11.1)*		
	Female:	0.9 *(0-4.8)*	0.9 *(0-4.2)*		
Aggression	Male:	0 *(0-1.2)*	0 *(0-1.5)*		
	Female:	0 *(0-0.3)*	0 *(0-0.7)*		
Hindes index		0.4 *(-1.2-1.4)*	0.3 *(-1.1-2.0)*		
Nose to nose		1.2 *(0-2.7)*	2 *(0-3)*		

Table 7: Model comparisons for the frequency of captive *A. nancymaae* female marking behaviors, including subcaudal, pectoral, and face marking, and urine washing. Also include the output from the model with the lowest AICc

Model Comparison

Fixed Effects	*Delta AICc*	*AICc Wt*	*K*
Phase * Group Type	0	0.68	6
Phase	2.79	0.17	4
Null	2.95	0.15	3

"Best" model output

Random effects	*Variance*	*SD*
Pair ID	2.12	1.46
Observer ID	3.11	1.76

	Estimate	*SE*	*z*	*Pr(>|z|)*	*Cumulative AICc weight*	*N models including variable*
Intercept	-2.96	1.07	-2.73	0.006	-	-
Phase (non-fecund)	-0.73	0.27	-2.75	0.006	0.85	2
Group Type (new)	-0.56	0.86	-0.66	0.510	0.68	1
Phase (non-fecund) + Group Type (new)	1.05	0.40	2.58	0.010	0.68	1

SE: standard error; SD: standard deviation

Table 8: Model comparisons for the frequency of captive *A. nancymaae* male genital investigations, and the output from the model with the lowest AICc

Model Comparison			
Fixed Effects	*Delta AICc*	*AICc Wt*	*K*
Phase * Group Type	0	0.36	6
Null	0.05	0.35	3
Phase	0.49	0.28	4

"Best" model output		
Random effects	*Variance*	*SD*
Pair ID	0.04	0.19
Observer ID	0	0

	Estimate	*SE*	*z*	*Pr(>|z|)*	*Cumulative AICc weight*	*N models including variable*
Intercept	0.30	0.13	2.23	0.03	-	-
Phase (non-fecund)	0.22	0.14	1.53	0.13	0.64	2
Group Type (new)	-0.20	0.20	-1.01	0.31	0.36	1
Phase (non-fecund) + Group Type (new)	-0.20	0.22	-0.92	0.36	0.36	1

SE: standard error; SD: standard deviation

Table 9: Model comparisons for the frequency of captive *A. nancymaae* female presents and approaches, and the output from the model with the lowest AICc

Model Comparison			
Fixed Effects	*Delta AICc*	*AICc Wt*	*K*
Null	0	0.43	3
Phase	0.4	0.35	4
Phase * Group Type	1.3	0.22	6

"Second best" model output		
Random effects	*Variance*	*SD*
Pair ID	0.62	0.78
Observer ID	0.01	0.08

	Estimate	*SE*	*z*	*Pr(>|z|)*	*Cumulative AICc weight*	*N models including variable*
Intercept	1.33	0.22	6.13	<0.001		
Phase (non-fecund)	-0.11	0.08	-1.27	0.2	0.57	2

SE: standard error; SD: standard deviation

Table 10: Differences in the frequency of behaviors (hourly rates) in newly-formed (8 pairs) and established (8 pairs) *A. nancymaae* pairs, excluding gestation

		Behavioral frequency: median (*range*)				
	Sex	**New Pair**	**Established Pair**	**Wilcoxon rank sum (W)**	**Effect size (r)**	**P value**
Copulations		0.1 (*0-1.1*)	0.3 (*0-1.0*)	29	-0.07	0.79
Genital inspections	Male:	0.71 (*0-1.1*)	1.7 (*0-4.2*)	40.5	-0.21	0.40
	Female:	0.1 (*0-1.1*)	0.5 (*0-1.4*)	46	-0.36	0.16
Presents	Female:	0.03 (*0-0.1*)	0.03 (*0-0.4*)	37	-0.13	0.61
Partner marking	Male:	0 (*0-2.3*)	0.15 (*0-3.8*)	42	-0.29	0.25
	Female:	0 (*0-1.6*)	0 (*0-2.6*)	31.5	0	1
Sniffing	Male:	3.6 *(2.7-14.9)*	2.8 *(0.9-19.8)*	22	-0.32	0.31
	Female:	3.2 *(2.2-3.9)*	2.4 *(0.8-5.3)*	13	-0.49	0.05
Subcaudal marking	Male:	0.2 (*0-5.3*)	0.4 (*0-10.2*)	33	-0.01	0.96
	Female:	0.3 (*0-6.3*)	0.3 (*0-4.6*)	31.5	0	1
Pectoral marking	Male:	0 (*0-0*)	0 (*0-0.12*)	3 events		
	Female:	0 (*0-0.3*)	0 (*0-0.2*)	3 events		
Face marking	Male:	0.22 (*0.10-0.5*)	0.18 (*0-0.4*)	23	-0.22	0.36
	Female:	0.6 (*0.1-1.4*)	0.2 (*0-0.4*)	20	-0.3	0.2
Urine washing	Male:	0 (*0-0.5*)	0 (*0-0.3*)	12 events		
	Female:	0 (*0-0.3*)	0.03 (*0-2.41*)	38 events		
Arching	Male:	0.4 (*0-0.9*)	0 (*0-1.1)*	20	-0.32	0.21
	Female:	0.3 (*0-2.0*)	0.3 (*0-1.7*)	28	-0.09	0.71
Hindes index		0.1 (*--0.4-0.6*)	0.2 (*-0.2-0.4*)	39	-0.17	0.51
Nose to nose		2.1 (*0.9-4.1*)	1.4 (*0.8-3.6*)	24	-0.19	0.44
Touching	Male:	1.5 *(0.3-2.5)*	1.6 *(0.3-7.0)*	36	-0.09	0.72
	Female:	0.5 *(0.4-2.3)*	0.8 *(0.2-2.4)*	36	-0.09	0.72
Approaches	Male:	19.3 *(6.1-30.1)*	21.3 *(2.8-41.7)*	35	-0.06	0.80
	Female:	15 *(4.5-35.0)*	*9.3 (4.2-24.7)*	21	-0.28	0.27
Grooming	Male:	0.8 *(0-4.2)*	0.3 *(0-1.1)*	17.5	-0.37	0.14
	Female:	0.5 *(0.1-0.9)*	0.2 *(0-0.9)*	26	-0.14	0.56
Aggression	Male:	0.04 *(0-1.0)*	0 *(0-0.2)*	24	-0.22	0.39
	Female:	0 *(0-0.1)*	0 *(0-0.2)*	41.5	-0.31	0.21

Figures

Figure 1: Hourly rates of copulations in the fecund and non-fecund phase (left), and in new and established pairs (right), of captive *A. nancymaae*

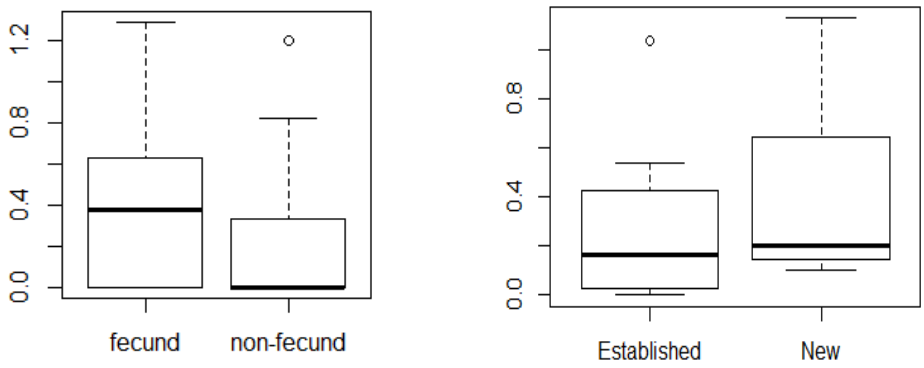


Figure 2: Hourly rates of genital inspections by the male in the fecund and non-fecund phase in 15 captive *A. nancymaae* pairs

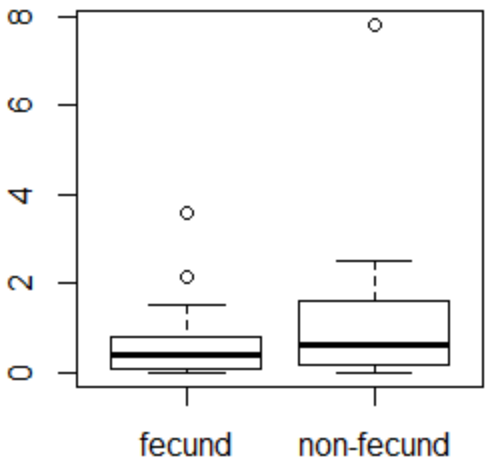


Figure 3: Hourly rates of captive male *A. nancymaae* partner-marking in the fecund and non-fecund phases (left) and in new and established pairs (right)

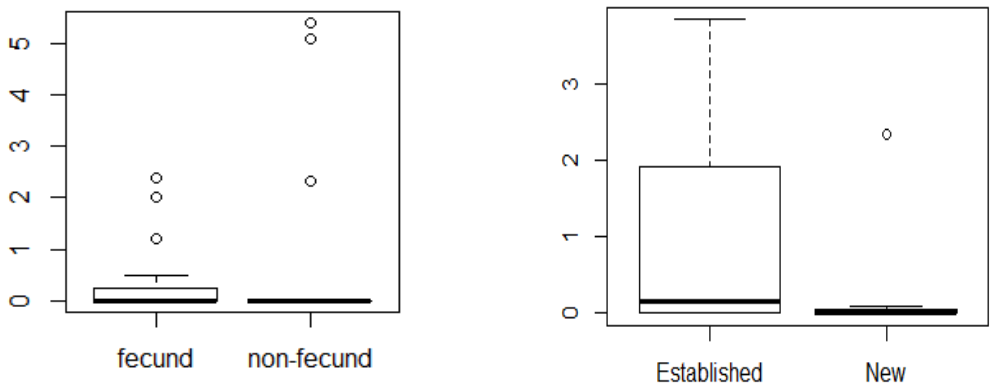


Figure 4: Hourly rates of arching behavior in the fecund and non-fecund phases by males (top left) and females (top right), and in new and established pairs by males (bottom left) and females (bottom right) of captive *A. nancymaae*

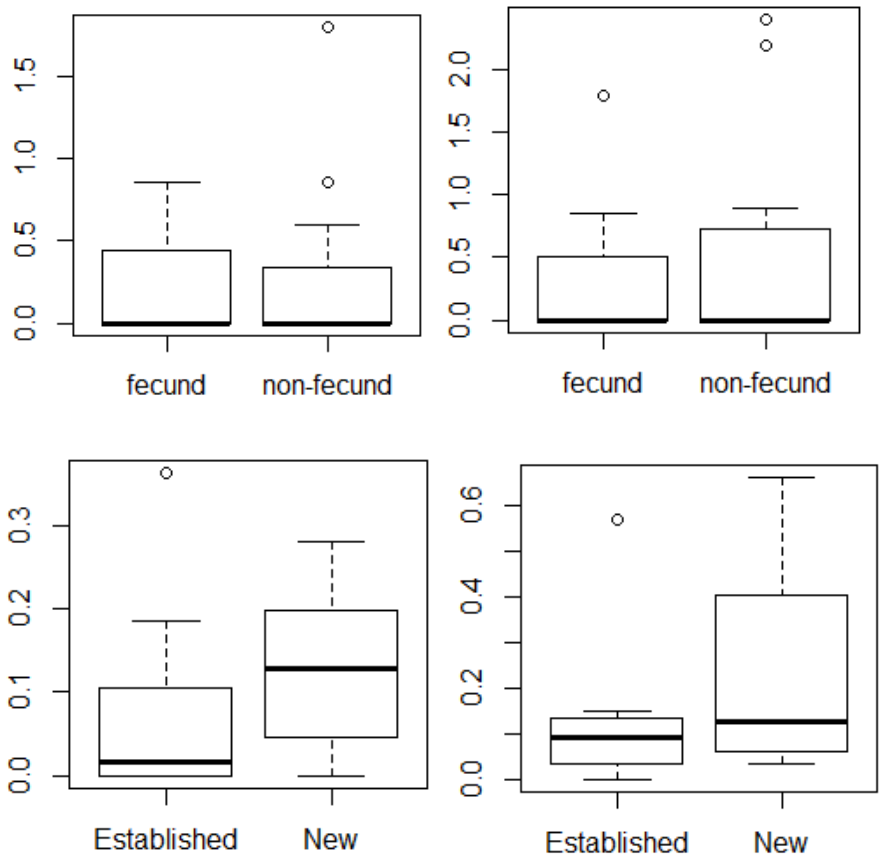


Figure 5: The number of copulations observed in wild *A. azarae* on the days before and after the estimated date of ovulation (Day 0)

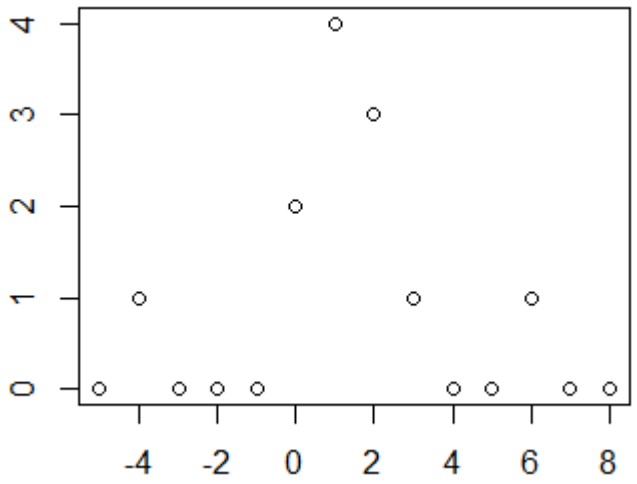


Figure 6: Hourly rates the female sniffed the male in new and established pairs of *A. nancymaae*

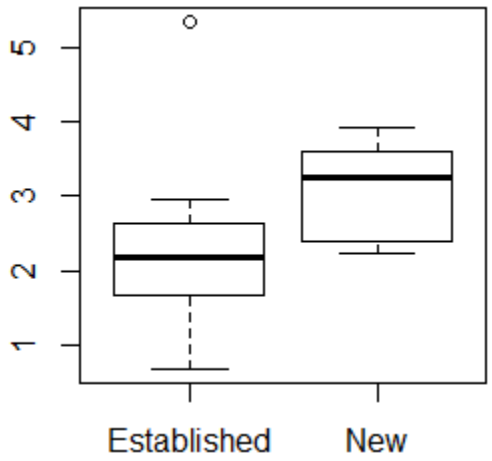
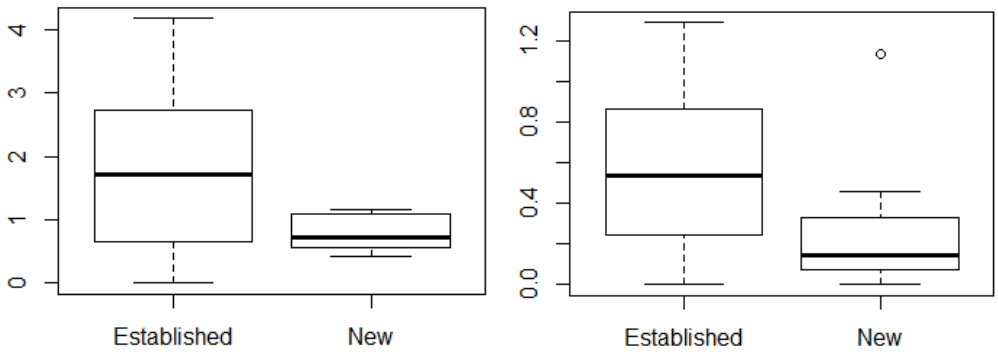


Figure 7: Hourly rates of males inspecting female genitals (left) and females inspecting male genitals (right) in established and new pairs of *A. nancymaae*



CHAPTER 5: Are olfactory traits in a pair-bonded primate under sexual selection? An evaluation of sexual dimorphism in *Aotus nancymaae*

Abstract

Sexual selection has seemingly influenced chemical communication in numerous non-human primates, although it is unclear whether this includes strictly pair-living and pair-bonded taxa. The physical characteristics of *Aotus* suggest that directional selection has not played a role in this taxon. However, given their nocturnality, owl monkey olfactory traits may show differing patterns of sexual selection than visual traits. If sexual selection has influenced chemical communication in *Aotus*, then we expect there to be larger scent glands and greater scent-marking in females given the high degree of paternal care, as it has been proposed for callitrichines. We evaluated sex differences in the qualitative and quantitative descriptions of the subcaudal and perianal glandular regions of male (n=40) and female (n=34) captive owl monkeys (*A. nancymaae*), and in the olfactory behaviors performed within breeding pairs (n=16). Males had larger areas of secretion retained in the hairs covering the subcaudal gland, whereas females had more and darker secretion than males covering the perianal region. Males inspected the genital region of their partners more frequently than females did, but the sexes did not differ much in other investigative and marking behaviors. The observed sex differences and variation in olfactory traits are consistent with the hypothesis that sexual selection has influenced chemical communication in *Aotus*. Still, contrary to our expectations, there was no evidence that females have larger glands or more frequently scent mark. Sex differences

of olfactory traits in *Aotus* were less extreme compared to other non-human primates showing olfactory dimorphism.

Introduction

The influence of sexual selection on olfactory communication was first proposed by Darwin (1871). Despite these early considerations, the study of olfactory communication in primates only began developing in the 1970s, and eventually dispelled the notion of the "microsmatic" primate (Heymann, 2006a; Laska and Salazar, 2015). With the growth in the study of primate olfaction, it has also become increasingly clear that sexual selection has influenced the evolution of chemical communication in non-human primates (Heymann, 2003a, 2006b; Snowdon, 2004; Drea, 2015; Setchell, 2016). This evidence is particularly compelling when considering it in the context of Snowdon's (2004) five criteria for identifying traits as sexually selected. More specifically, Snowdon (2004) proposed that, for a trait to be considered as sexually selected, it is necessary to show that 1) it is sexually dimorphic, 2) it varies within a population, 3) individuals discriminate between variants of the trait, 4) individuals show preference, related to reproduction, for a particular variant, and 5) individuals have differential reproductive success that is related to variation in the trait**.** Among non-human primates, there is solid evidence showing that sexual dimorphism and variation are present in chemical (the odor and composition of odor), physical (e.g. scent glands), and behavioral (e.g., scent-marking) olfactory traits (Snowdon, 2004; Drea, 2015; Setchell, 2016). Individuals can discriminate between odors and display a preference for particular variants (Scordato and Drea, 2007). Finally, at least for some taxa, certain variants of olfactory traits seem to be related to reproductive success, as is the case with olfaction-mediated reproductive suppression of adult callitrichid females (Ziegler, 2013b).

The sense of smell has been relatively neglected in anthropology when compared, for instance, to vision (Hoover, 2010). This is despite the rich history of olfactory research in humans, which suggests putative chemosignals may aid in mediating social relationships by influencing mood, hormones, and possibly even mate choice (Wysocki and Preti, 2004; Lübke and Pause, 2015). Given the potential for these putative chemosignals to modulate human relationships, and in light of sex differences in the ability to perceive odors (Brand and Millot, 2009), neural responses to odors (Savic et al., 2001), and the olfactory bulb (Oliveira-Pinto et al., 2014), it is reasonable to consider that sexual selection may have had a role in their evolution.

Non-human primates are valuable models to explore the possible role of sexual selection on the evolution of olfactory traits. Understanding olfactory traits and the way that they function in extant non-human primate taxa will broaden our base of knowledge with which to interpret studies of modern humans and reconstructions of early human behavior. To conduct meaningful comparisons, it is necessary to gather data from a range of non-human primate taxa, which display varying degrees of sexual dimorphism and different social and mating systems. Evidence of sexual selection in olfactory traits may not only vary across species, but the degree and/or direction of selection on olfactory traits may differ from other sexually selected traits within a species. In some cases, such as the sexually dimorphic mandrill, it is clear that sex differences in olfactory traits parallel those observed in other traits (Setchell, 2016). On the other hand, among the "monomorphic" sifakas (*Propithecus spp.*), stabilizing selection favors intermediate body size in males and females (Lawler et al., 2005); even so, scent-marking rates and the presence of scent glands differ between the sexes (Schilling, 1979; Lewis, 2005; Pochron

et al., 2005), possibly in response to directional sexual selection. In taxa where chemical communication contributes to mating opportunities, even those seemingly "monomorphic" taxa, may show evidence of intra- or inter-sexual selection within the suite of olfactory traits. The potential discrepancy between the degree and direction of sexual selection acting on olfactory and visual traits underscores the need to produce a broad representation of the way that chemosignals function across the range of physical dimorphism and types of social and mating systems.

Owl monkeys have an atypical social and mating system that differs from any other non-human primate taxa on which chemical communication studies have been conducted. Owl monkeys are strictly pair-living and there is no evidence of extra-pair paternity in the only wild population where this has been assessed (Huck et al., 2014). Additionally, like humans, male and female owl monkeys form stable and long-lasting relationships ("pair bonds") and participate jointly in the care of the young (Fernandez-Duque, 2012). Male and female owl monkeys experience similar levels of intra-sexual competition for mates because adults of both sexes are at high risk of being evicted and replaced from their breeding groups by challenging, solitary, floaters (Fernandez-Duque and Huck, 2013). In support of the proposition that they experience similar levels of intra-sexual competition, they show extremely low levels of dimorphism in body size, coloration, and other body measurements, with the exception of hindlimb and canine length (Fernandez-Duque, 2011; Huck et al., 2011). Even the external genitalia can be remarkably similar (Figure 1).

However, males and females, while both investing heavily in infant care, are extremely dimorphic in the type of care offered. Females limit their direct care primarily

to nursing infants, whereas males provide the vast majority of all other types of direct care such as transporting and sharing food with them (Dixson and Fleming, 1981; Wright, 1984; Rotundo et al., 2005). Therefore, while competition for mates may not differ much between the sexes in this taxon, biparental care may influence the degree and direction of sexual selection. In this regard, Heymann (2003a) has proposed that the degree of male care will influence sexual selection of chemical communication among platyrrhines, so that where males provide extensive infant care, competition for these males will drive relatively more elaborate female traits. Specifically, he predicted that in taxa where males provide greater care, olfactory traits will be female-biased (with females having larger scent glands than males and higher rates of scent-marking), whereas the reverse will occur if females are the primary care-givers (Heymann, 2003a). Patterns of infant care, scent-marking, and gland size are generally consistent with this hypothesis among some New World monkeys, although scent-marking rates of *Aotus* in support of this hypothesis were extremely limited (Heymann, 2003b).

Owl monkeys seem to rely heavily on chemical communication, making them an excellent model to investigate whether sexual selection has influenced olfactory traits differently than other traits. They possess a specialized subcaudal gland that produces a chemically rich secretion which encodes sex information (Spence-Aizenberg et al., accepted). Both males and females, in the wild (Corley et al., 2014) and in captivity (Wolovich and Evans, 2007), engage in scent-marking and social sniffing of partners, indicating that secretions and odor play a role in inter-sexual communication. This is reinforced by captive research demonstrating that the reduction of the reception of olfactory cues reduces aggressive interactions between unfamiliar males (Hunter and

Dixson, 1983), which suggests that odor plays a role in intra-sexual competition among males. Odors from subcaudal secretions likely serve a reproductive purpose given that the gland develops with age (Dixson et al., 1981; Huck et al., 2011) and reproductive maturity. These behaviors, coupled with their nocturnal activity patterns, make it very likely that chemical communication could be more directly affected by sexual selection than visual cues such as coloration or body size.

We hypothesized that chemical signaling, as an integral component of inter-sexual communication in *Aotus*, has been influenced by sexual selection. We began to examine this hypothesis with a qualitative and quantitative examination of physical and behavioral olfactory traits in captive Nancy Ma's owl monkeys (*Aotus nancymaae*). Our first objective was to provide the first systematic description of the subcaudal gland and perianal regions. The subcaudal gland is a field of hypertrophic sebaceous and apocrine glands covered with thicker and more densely planted stiff, specialized hairs (Hill et al., 1959; Hanson and Montagna, 1962). Hairs overlying the subcaudal gland may split at the terminal ends, producing a felted appearance (Hill et al., 1959). The perianal region is a hairless region between the genitals and the base of the tail that has larger apocrine and sebaceous glands than most of the skin (Hanson and Montagna, 1962). Specifically, we describe the subcaudal gland size, the felting of the hair covering the subcaudal gland, and the color and amount of secretion produced in the perianal region.

Secondly, we evaluated whether there was evidence of sexual selection in physical and behavioral olfactory traits. Using the framework for identifying sexually selected traits developed by Snowdon (2004), we assessed the first two criteria: a) that a trait is sexually dimorphic, and b) it varies within a population. Using a number of individuals

large enough to evaluate sex differences and population variation, we compared the subcaudal gland size and felting of subcaudal hair, the color and amount of perianal secretion, marking, and investigative behaviors between male and female owl monkeys. Dimorphism in these traits would be consistent with the hypothesis that there have been differential selection pressures operating on males and females. Finally, we assessed whether the levels of dimorphism observed in the physical and olfactory traits are consistent with the hypothesis proposed by Heymann (2003a). Given the high degree of paternal care in *Aotus*, we predicted females will have larger subcaudal glands and higher rates of scent-marking.

Methods

Subjects

We collected data from *A. nancymaae* individuals housed at the Owl Monkey Breeding and Research Resource (OMBRR) located in the Keeling Center for Comparative Medicine and Research (MD Anderson Cancer Center, University of Texas, Bastrop) in 2013 and 2015. The OMBRR houses approximately 400 owl monkeys on a semi-reversed light cycle with periods of darkness extending approximately from 1500h to 0000h. Animals were housed in pairs or family groups in enclosures approximately 1.8 m^3 in volume. They were fed primate biscuit and fruit twice daily before 1500h, and food was available throughout the dark cycle. Although enclosures were directly adjacent to one another, the animals were visually isolated from each other, and white noise produced by a waterfall buffered the acoustic interactions within the room.

Gland Appearance Data Collection

We obtained gland measurements and appearance information of 74 individuals (40 males, 34 females) from photographs taken in August 2015. Six individuals were subadults (24.1-48 mos) and 68 were adults (>48 mos) following age classifications used for wild owl monkeys (Huck et al., 2011). We analyzed subadults and adults together because these two age categories are not reliably distinguished chemically in this population (Spence-Aizenberg et al., accepted), and subadult pairs are reproductively active and able to conceive (Spence-Aizenberg et al., unpublished data). On average, females were slightly older than males (Table 1). We took at least two photographs per individual while animals were manually restrained for monthly physicals; a tape measure held next to the perianal and subcaudal region in the photograph provided a scale for measurement. One observer (ASA) calculated the surface area of the gland (cm^2) in ImageJ (Schneider et al., 2012) using the freeform shape to outline the gland (Figure 2). Within the gland area, visually identified as exhibiting thicker, stiff, discolored hairs, we took two different measurements: 1) the area of subcaudal hairs that were wet with secretion ("gland secretion area", Figure 2), and 2) the area of subcaudal hair that was noticeably discolored from the remainder of the tail ("gland hair area", Figure 2), which encompassed the gland secretion section. We measured and outlined the surface area of each section three times per individual, and we report mean values for each individual. When we could not define a distinct area, we did not take measurements of the gland secretion (N=12) nor gland hair (N=5) areas. We scored felting of the subcaudal hairs as "yes" if the hairs were visibly split in an individual's photograph and "no" if they were not. If an individual could not be definitely identified as having split hairs or not, we excluded him/her from this analysis (N=27).

Using these photographs, we also collected information on the appearance of the perianal region. We recorded qualitative descriptions on a scale of one to three, excluding those individuals that we were unable to score due to the presence of urine or feces. We coded perianal secretion color as either very light in color with clear or yellow hue (1), a medium/orange tinted hue (2), or a dark/brown tinted hue (3) (N=34 females, 33 males; Figure 3). We quantified the amount of perianal secretion as the portion of perianal skin covered in secretion; secretion was scored as less than 25% (1), between 25%-75% (2), and greater than 75% coverage (3) (N=35 females, 33 males; Figure 4).

Behavioral Data Collection

We collected behavioral data from 16 breeding pairs; eight in 2013 and eight in 2015. Males and females wore colored collars so that observers could positively identify individuals. We collected all behavioral data during 20-minute focal periods following the sampling procedures and ethogram detailed in the Monogamous Primate Project protocols (Spence-Aizenberg et al., 2016), and modified for captive owl monkeys to focal-dyad sampling to simultaneously record the behavior of both the male and the female (Wolovich and Evans, 2007). During each focal sample, we used all-occurrence sampling of olfactory behaviors (Table 2) in 10 two-minute intervals. We recorded behaviors using a digital recorder, we then transcribed and transferred them to the database. We collected 694 focal samples; representing 231.3 hours of watching pairs, or 462.6 hours of individual "monkey-hours." We collected more focal samples in 2015 than in 2013, but within each season, the hours of observation were relatively balanced across pairs (2013: range 8-12 hrs, 2015: range 17-20 hrs). For the analyses, we used

average individual values for each behavior so that each individual contributed equally to it (see below).

Inter-Observer Reliability

The first author (ASA) and four research assistants collected the behavioral data. Observers trained together on each behavior and collected inter-observer reliability trials with at least two other observers. Agreement for overt behaviors (e.g. approaches and leaves) was high (~90%), whereas it was markedly lower for olfactory behaviors (range ~0% - ~60%). To understand the possible causes of this lower reliability, we visually inspected the behavioral frequencies reported by each observer independently; we found that similar patterns of sex biases emerged across observers. For example, the ratio of male to female genital inspection across observers ranged from 2.2-5.3, but mean female inspection rates were always lower than male rates for all observers. The stability of these patterns across observers suggests that overall frequencies of male and female behavior were accurately recorded, even though agreement on particular behavioral events may be low. We concluded that this lower reliability was primarily due to the subtle nature of many of these behaviors and the differences in visibility between observers when simultaneously watching the animals from slightly different positions. Therefore, we used focal samples collected by all observers.

Statistical Analyses

We used descriptive and non-parametric statistics due to the non-normal distribution of the gland size measurements and behavioral data, and the ordinal nature of the perianal

data. For each focal sample, we calculated the total number of times each olfactory behavior was exhibited by a male or a female, and then averaged it across all focal samples collected for each individual. We then multiplied these mean values by three to obtain the average hourly rate for each behavior. We used these individual average hourly rates in all statistical analyses.

In order to evaluate sex differences in gland appearance, we estimated and report median values and ranges of males and females for scent gland size, age, body mass, perianal secretion color, perianal secretion color, and olfactory behaviors. We examined sex differences using Wilcoxon rank sum tests and Pearson's Chi-squared tests. For all Wilcoxon tests, we calculated the effect size "r", using the "rFromWilcox" function (Field et al., 2012). Additionally, to better understand how these variables may interact with one another, we tested for correlations between gland size, perianal secretion color and amount, and age. We excluded body size as the average sex difference in mass was only 31g (Table 1). We conducted all statistical analyses in R (R Development Core Team, 2016).

Results

Subcaudal and Perianal Region

Males had subcaudal glands that were, on average, 1.3 times the size of the females' glands. The difference between the sexes was apparent when considering both the gland hair and the gland secretion areas (Table 1). There was virtually no relationship between the age of the individual and the size of the gland hair area (Spearman rho: -0.128, p=0.32, n=64) or the gland secretion area (Spearman rho: -0.024, p=0.84, n=64).

Additionally, sex differences in body mass were negligible (Table 1). The surfaces of the gland hair and gland secretion areas showed a moderate positive relationship (Spearman rho: 0.532, $p<0.001$, n=64). Given this moderate association, we limited the analyses below to the size of the gland as measured by the gland secretion area, for which we have more individuals measured. We observed felting in 52% of males (N=12/23) and 29% of females (N=7/24); the difference was not statistically significant (Pearson's chi-square with Yates' continuity correction: $X^2=1.7145$, df=1, P=0.190).

Males and females also showed noticeable differences in the appearance of the perianal region. Females displayed darker secretion than males. Most females (88%, 30/34) scored a 2 or higher value, whereas most males (79%, 26/33) scored a 2 or lower value (Table 3). There was also a tendency for females to have more oil covering their perianal region than males did. Nearly half of the females (46%, 16/35) had greater than 75% of the perianal region covered with secretion, whereas nearly half of the males (45%, 15/33) had less than 25% of the perianal region covered in secretion, although the difference was not statistically significant (Table 3). The correlation between perianal secretion color and amount was small (Spearman's rank correlation, rho= -0.19, S=57165, P=0.120, n=66).

Perianal secretion color increased with age (rho= 0.32, S=29561, P=0.009). On the other hand, increases with age in the amount of secretion and gland size were minimal (secretion amount: rho= -0.05, S=47916, P=0.7; gland size: rho=0.01, S=56486, P=0.9). Gland size was not strongly correlated with the color (rho=0.10, S=35682, P=0.4) nor the amount of secretion present on the perianal region (rho=0.05, S=39410, P=0.67).

Olfactory Behaviors

Males and females showed similar levels of all marking behaviors (Table 4). Females tended to do more subcaudal scent-marking and urine washing; males tended to partner-mark more frequently than females (Figure 5). Males and females showed even greater similarities in scent-marking with the pectoral gland or face, although none of the sex differences in marking behaviors were statistically significant.

Males engaged in all investigative behaviors more frequently than females (Table 4). Most notably, males engaged in genital inspections of their partner four times as frequently as females did (Figure 5). Sex differences in partner and object sniffing were comparatively smaller, and not statistically significant, with the sniffing of objects being the least dimorphic of the investigative behaviors.

Subcaudal scent-marking was the most frequent marking behavior, with hourly rates eight and 14 times greater than those for marking with the pectoral gland in males and females respectively (Table 4). Partner sniffing was more frequent than any other investigative behavior, occurring approximately 2.5 times more than sniffing objects, and three to ten times more than genital sniffing.

Discussion

Our evaluation of physical and behavioral olfactory traits in owl monkeys (*Aotus nancymaae*) shows sexual dimorphism and intra-sexual variation in some of these traits. These results add to our earlier findings of sex differences in the chemical components of glandular secretions (Spence-Aizenberg et al., accepted). Together, the data fulfill the first two criteria of Snowdon's (2004) framework for identifying sexually selected traits.

They also provide preliminary support for the hypothesis that sexual selection has influenced the evolution of olfactory communication in owl monkeys, as has been proposed for other non-human primates (Heymann, 2003a; Snowdon, 2004; Drea, 2015; Setchell, 2016). Our results also suggest that the patterns of sexual dimorphism in olfactory traits do not differ from other physical traits in *Aotus* as much as have been reported for other "monomorphic" taxa such as sifakas (Schilling, 1979; Lewis, 2005; Pochron et al., 2005) and tamarins (Heymann, 2003b). This result is perhaps expected given the presumably equal levels of mating competition in *Aotus*, though it contrasts with the degree of male care.

We did find seemingly important sex differences in subcaudal gland size. Male owl monkeys had larger areas and greater variation in the size of the subcaudal gland, as measured by the hairs covering the gland that were coated with wet secretion, than females. They also showed more felting of the hairs than females did. The greater range in variation of subcaudal gland size in males parallels the patterns of intra-sexual variation in the chemical profiles of these glandular secretions, with the profiles of males varying more than those of females (Spence-Aizenberg et al., accepted). It also confirms earlier, unquantified, reports that males have more developed subcaudal glands than females (Hill et al., 1959), and estimates of gland size from a wild population of *A. azarae*, which show that median stained areas of the subcaudal gland are approximately 1.2 times larger in adult males than females (Huck et al., 2011).

Secretion covering the perianal region also differed between the sexes. Females displayed darker secretion and tended to show greater amounts of secretion covering the perianal skin. This region has received little attention in the literature, but its potential

importance for olfactory communication should be considered. When pairmates inspect the anogenital area, it is extremely likely that the glandular secretion on the perianal region contributes to the perceived odor. Whether or not the secretion from the perianal region accumulates in the hairs of the subcaudal gland, or are deposited in scent marks, is unknown. Likewise, the extent to which these secretions differ chemically from those in the subcaudal region is unclear. Our observations are similar to those described for cotton-top tamarins in which the glands of females are more oily and more pigmented than those of males (French and Cleveland, 1984). Sex differences in the color of glandular secretions have also been reported in badgers (Buesching et al., 2002), beavers (Schulte et al., 1995), and aardwolves (Sliwa, 1996). In aardwolves, sex differences in the color of secretion may be caused by brown pigment granules in the secretory cells, which are present in males, but absent in females (Stoeckelhuber et al., 2000). Still, the relevance of these differences is unclear.

Investigative behaviors did differ between males and females, with male owl monkeys investigating the anogenital region of their partner more often than females did. Other investigative behaviors (object sniffing and partner sniffing) were also more frequently done by males, but to a lesser extent. That the greatest sex differences were observed in inspections suggests that they are not just a byproduct of sniffing behavior being generally more frequent in males. Instead, it suggests this behavior is likely socially or sexually motivated, and biologically meaningful, with males showing greater olfactory interest in their partners than the environment. In contrast, we found similar levels of scent-marking in males and females, with a slight bias towards greater subcaudal marking by females. The lack of strong sex differences in marking of

substrates and strong male bias in genital sniffing confirm what has been reported in another captive population of *A. nancymaae* (Wolovich and Evans, 2007). However, unlike this previous study, we observed females engaging in partner-marking and did not find a noticeable sex difference in urine washing. Additionally, while our data show a slight bias in subcaudal marking by females, the opposite pattern was previously observed (Wolovich and Evans, 2007).

Overall, the patterns of sex differences across the suite of *A. nancymaae* olfactory traits that we investigated are not consistent with Heymann's (2003a) hypothesis that the degree of male care influences the direction of sexual selection on chemical communication. While some aspects of chemical communication seemed to be more frequent among females, the size of the subcaudal gland size was larger in males. These findings differ greatly from patterns of olfactory behavioral and physical traits in other taxa where males are more heavily involved in infant care. For example, tamarin females have larger scent glands (Epple et al., 1982; French and Cleveland, 1984) and engage in more frequent scent-marking (French and Cleveland, 1984; Heymann, 1998; Smith and Gordon, 2002) than males. Instead, the larger size and greater variation of gland size in male *A. nanycmaae* supports the idea that there has been more selection for large subcaudal gland size on male than female owl monkeys.

In contrast, the perianal region shows slight female bias in the production of secretion, and the variation in color underlines the sex differences in the secretions. A larger surface area of the subcaudal gland could allow for greater secretion production and certainly for greater surface area to hold the secretion. If individuals can produce more secretion, then they might be able to deposit more scent marks, or retain more

secretion in the subcaudal hairs, potentially producing stronger odor signals than individuals with smaller subcaudal glands. It is also possible there is some sex-specificity in the importance of these two glandular areas. However, without knowing whether the secretions emitted from these two glandular areas are chemically similar or different, it is impossible to know what the function of these may be or how they might differ between the sexes.

Our data on behavioral olfactory traits suggest that it is the female signals that are of greatest interest within pairs. Males spent more time investigating females than females investigating males, and there was a slight tendency for females to subcaudally mark more frequently than males. We interpret this as possibly implying that males are investing more time into actively perceiving female olfactory signals than females are from males. It is also possible that information encoded in female secretions presents information more useful for intra-pair communication than do male secretions, and seems plausible to suggest that reproductive status (see Chapter 4), or fecundity, is signaled in glandular secretions or other sources of olfactory signals (such as urine), as is observed in callitrichines (Ziegler et al., 1993; Converse et al., 1995) and lemurs (Scordato and Drea, 2007).

Our data implicate the subcaudal and/or perianal region as the most integral to chemical communication within breeding pairs. We found that scent-marking with the perianal/subcaudal region was more frequent than scent-marking with the face or pectoral gland. The behavioral data are in agreement with the anatomy findings since the subcaudal gland is more developed, larger, and secretes more. Furthermore, the specialization of the hairs and the extreme subcaudal position of the gland when

compared to the location of scent glands in other platyrrhines (Hill et al., 1959) suggests that intensive selection pressures have led to the development and maintenance the subcaudal gland in owl monkeys.

Overall, the patterns of sexual dimorphism in physical and behavioral olfactory traits are compatible with the proposition that there may have been differing directional selection pressures on males and females regarding olfactory communication. Given that some potential functions of olfactory communication in owl monkeys, such as territory defense or facilitating a bond between pairmates, would not necessitate sex differences in physical or behavioral olfactory traits, it seems likely that the sex differences we observed are driven by sexual selection. To further explore this possibility, future research evaluating Snowdon's (2004) third, fourth and fifth criteria should be conducted. Behavioral bioassays in captive populations can be used to evaluate whether individuals can discriminate between odors, and whether there are preferences for a particular variant (see Chapters 3 and 4). To complement what can be learned from experimental manipulations in the laboratory, long-term research in wild populations should explore the relationships between chemical, physical, or behavioral olfactory traits, pair bond dynamics and reproductive success. Additionally, future work looking at sex differences in the olfactory bulbs, vomeronasal organs, or processing of odors in owl monkeys and other non-human primates could inform how sexual selection may have influenced the perception of chemosignals (Heymann, 2006b). Finally, our study shows that strictly pair-living non-human primates, with little to no sexual dimorphism in most physical traits, do show some degree of dimorphism in olfactory traits possibly indicative of directional selection. Thus, our study contributes to expanding knowledge of the

relationship between chemical communication and sexual selection in non-human primates, which can ultimately facilitate a better understanding of the evolution of chemical communication in humans.

Tables

Table 1: Number of individuals (N), medians (ranges), effect sizes, and statistical tests of differences in the age, body mass, and subcaudal gland size between male and female *A. nancymaae*

Measurement	Sex	N	Median (*range*)	Effect Size (r)	Wilcoxon Rank Sum (W)	P Value
Age	female	34	7.5yrs (*3.5-15.1*)	-0.21	852	0.06
	male	39	5.6yrs (*3.4-16.1*)			
Body mass	female	36	957g (*802 - 1336*)	-0.02	702.5	0.86
	male	40	988g (*786 - 1318*)			
Gland hair area	female	26	3.3cm^2 (*1.6 - 5.9*)	-0.46	230	<0.05
	male	38	4.4cm^2 (*2.2 - 8.7*)			
Gland secretion area	female	32	2.2cm^2 (*0.4 - 5.0*)	-0.23	453	<0.05
	male	39	3.0cm^2 (*0.8 - 7.7*)			

Table 2: Ethogram of the olfactory behaviors observed

Behavioral Category	Behavior	Description
Marking	Subcaudal scent-marking	The subcaudal region is in contact with a substrate and the body is slid forward or laterally moving the rear part of the body
	Pectoral scent-marking	The chest region is moved with pressure and friction against the substrate by sliding the body forward. It may also be pressed in a downward motion with hands and/or arms
	Face scent-marking (muzzle rub)	The face is in contact with a substrate and the cheek is slid forward or laterally against the substrate
	Partner-marking	Rubs subcaudal and/or anogenital area on another individual
	Urine washing	Hands are wet with animals own urine and then rubbed on some part of its body
Investigative	Genital sniffing	Sniffing, licking, or exploring the anogenital area, or the urine of partner
	Partner sniffing	Place mouth on, or very close (<1 cm), to their partner's body, excluding the anogenital area, but is not grooming
	Object sniffing	Placing nose very close (<1 cm), touching, or licking an object

Table 3: Scores and statistical tests of sex differences in perianal secretion color and amount in *A. nancymaae*

	Sex	Score 1*	Score 2**	Score 3***	Pearson's Chi-square	P Value
Secretion Color	female	4	15	15	9.88	0.007
	male	15	11	7		
Secretion Amount	female	9	10	16	3.05	0.22
	male	15	8	10		

*color = light/yellow, amount = <25% coverage; ** color = medium/orange, amount = 25-75% coverage; ***color = dark/brown, amount = >75% coverage

Table 4: Medians (range), effect sizes, and statistical tests of differences in hourly rates of olfactory behaviors in male and female *A. nancymaae*

Behavior Type	Behavior	Female: median (range)	Male: median (range)	Effect Size (r)	Wilcoxon Rank Sum (W)	P Value
Marking	Subcaudal scent-marking	2.48 (0-17.14)	1.44 (0-10.30)	-0.15	144	0.559
	Pectoral scent-marking	0.03 (0-0.11)	0.03 (0-0.11)	-0.01	129	0.978
	Face scent-marking	0.18 (0-1.50)	0.18 (0-0.56)	-0.06	134.5	0.821
	Partner-marking	0 (0-1.80)	0 (0-3.96)	-0.13	115	0.618
	Urine washing	0.58 (0-2.35)	0.16 (0-0.50)	-0.21	147.5	0.398
Investi-gative	Genital sniffing	0.25 (0-1.34)	0.99 (0-4.13)	-0.63	61	0.012
	Partner sniffing	2.61 (0.73-5.04)	3.35 (1.06-19.64)	-0.30	95.5	0.228
	Object sniffing	0.98 (0.08-2.41)	1.34 (0.08-7.25)	-0.08	119	0.749

Figures

Figure 1: External genitalia of two male (top) and two female (bottom) *A. nancymaae* exemplifying the visual similarities between some males and females

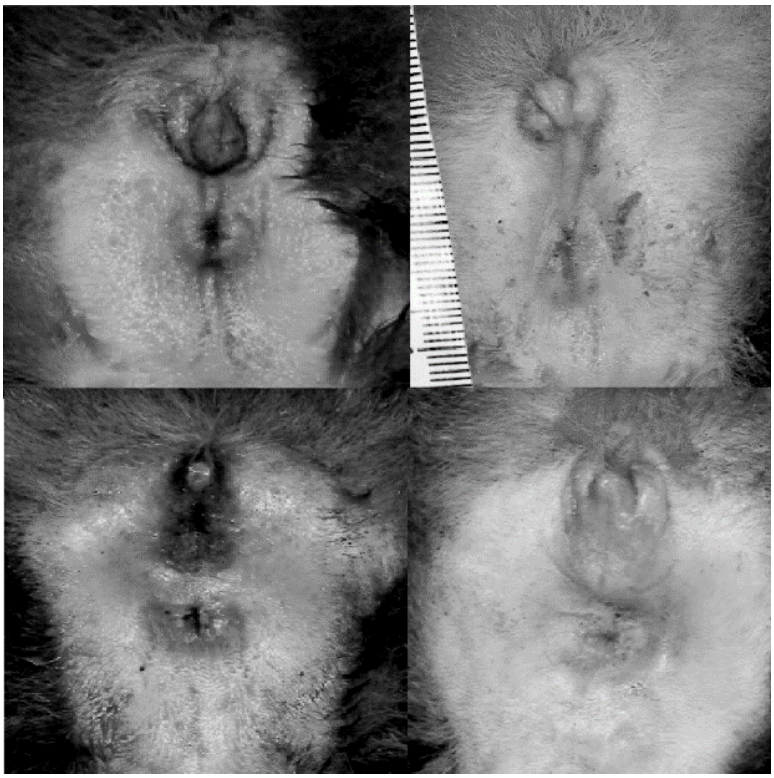


Figure 2: Outlines of the gland size as measured by the gland secretion area (left) and gland hair area (right) of an adult male *A. nancymaae*

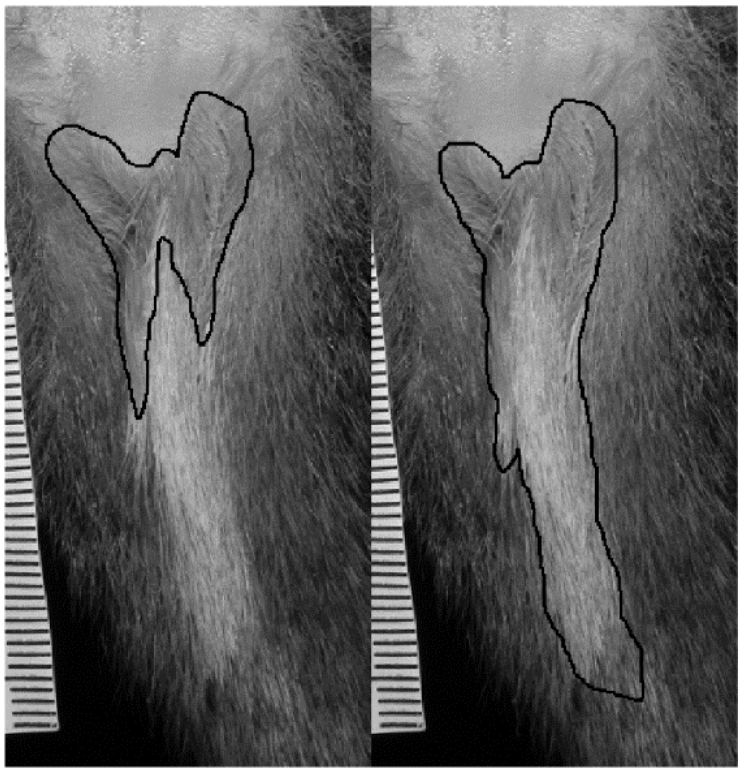


Figure 3: Variation in the color of the perianal secretion in *A. nancymaae*, with light/yellow (left) and dark/brown (right) seen on a male (left) and female (right)

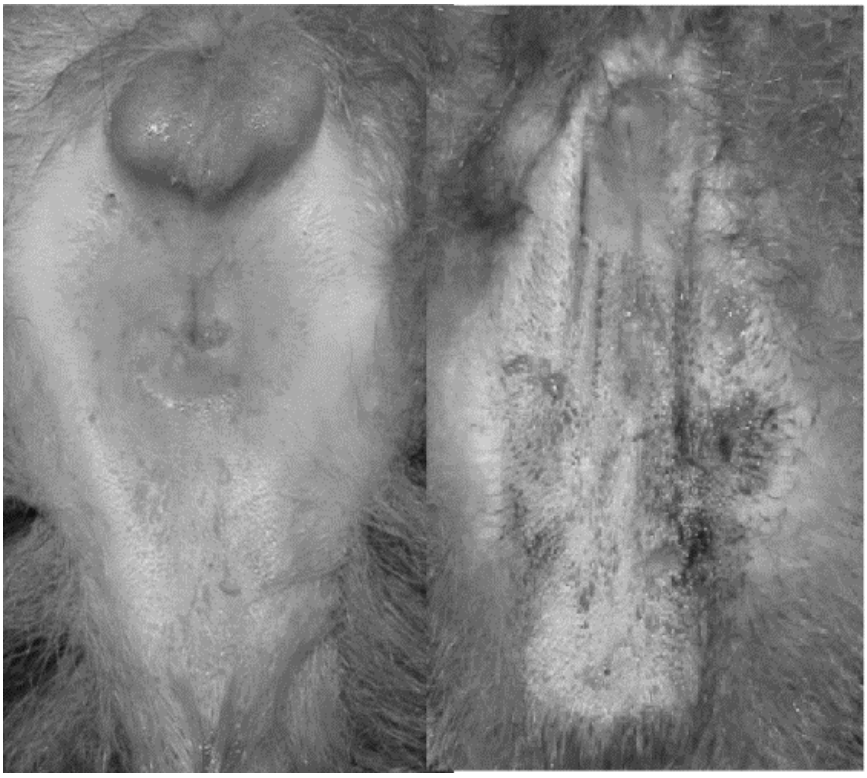


Figure 4: Example of a perianal region with a small amount (left) and a lot (right) of oil in a male (left) and female (right) *A. nancymaae*

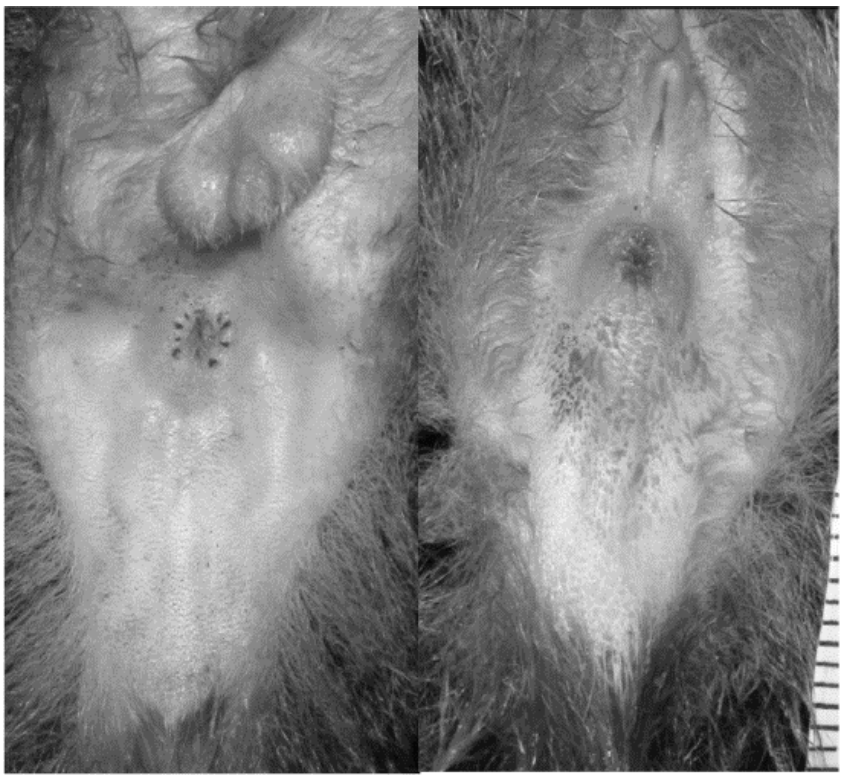
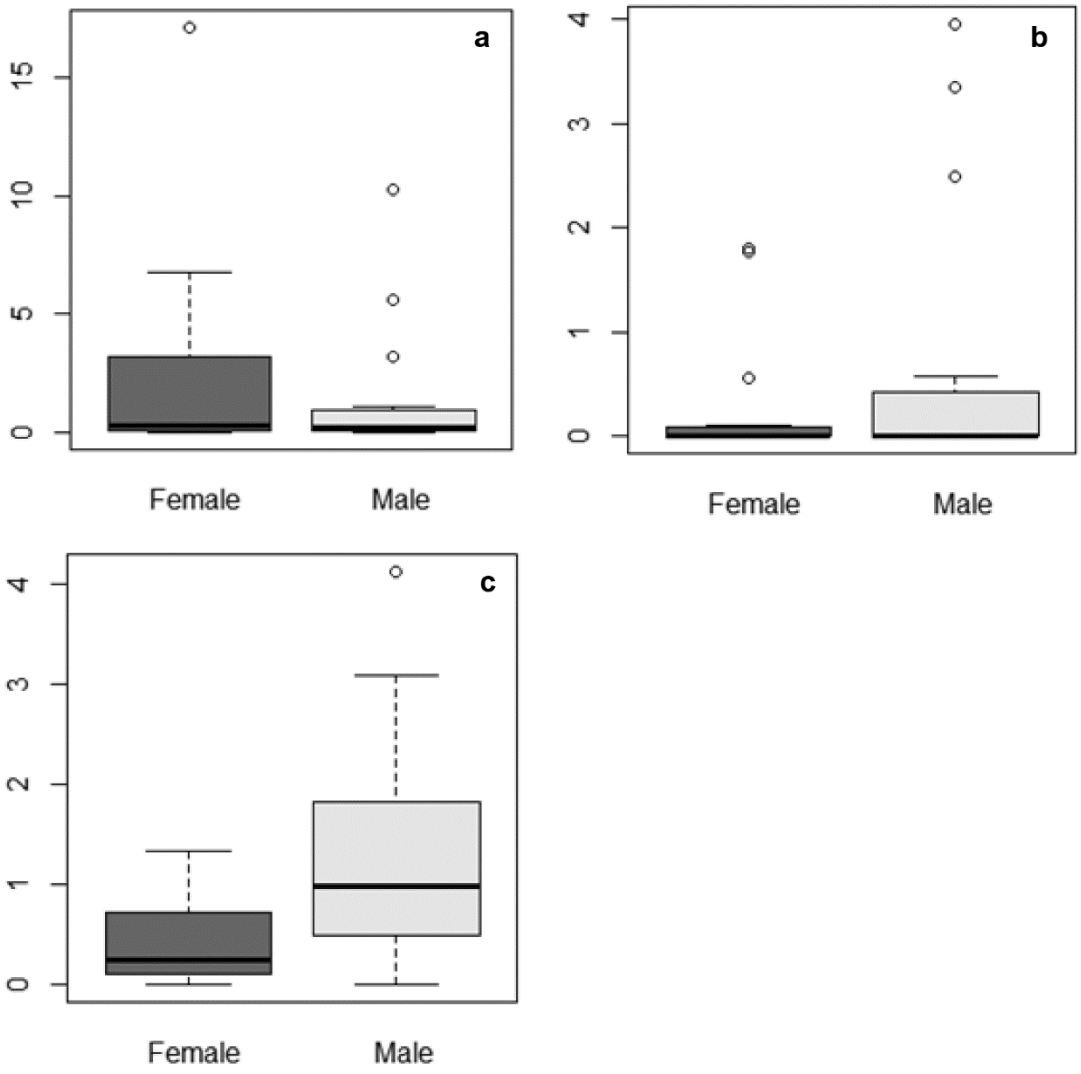


Figure 5: Mean hourly rates of subcaudal marking (a), partner marking (b), and anogenital inspections (c) in male and female *A. nanycmaae*



CHAPTER 6: Conclusion

In this thesis, I have conducted a comprehensive analysis of olfactory communication in owl monkeys (*Aotus* spp.), a nocturnal South American monkey. I explored the hypothesis that olfactory cues are used to communicate with potential mates by conducting a chemical analysis of glandular secretions produced by wild and captive owl monkeys in Chapter 2. In Chapter 3, I investigated the hypothesis that owl monkeys have chemosignals of relatedness by conducting behavioral bioassays in captivity, and through observations of relatedness between male-female pairs in a wild population. I explored whether owl monkey females produce chemosignals of fecundity using an experimental and observational approach in Chapter 4. Finally, in Chapter 5, I evaluated the degree of sexual dimorphism present in traits associated with olfactory communication to consider how sexual selection may have influenced olfactory communication in owl monkeys.

Chemical Components of Glandular Secretions

The research conducted in this thesis strongly shows that platyrrhine glandular secretions are chemically rich, encoding information likely used to signal to others. I found that two different owl monkey species *(Aotus nancymaae* and *A. azarae)*, living in differing environments, encode biologically relevant information in their glandular secretions. The chemical analyses of volatile compounds showed that there are putative chemosignals for sex, age, individual identity, gland type, and housing encoded in their secretions. Signals of sex were strong in both captive *A. nancymaae* and wild *A. azarae*,

and the signals of age were as strong as those of sex in wild *A. azarae.* These findings confirm that owl monkeys show evidence of chemosignals similar to what have been reported in strepsirrhines (Scordato et al., 2007; Morelli et al., 2013; Greene and Drea, 2014) and at least one catarrhine (Setchell et al., 2010; Vaglio et al., 2016) species. This represents the first chemical analysis of glandular secretions in a wild platyrrhine population, and of a captive primate population with the largest sample size to date.

Detection of Chemosignals

The use of behavioral bioassays in this dissertation not only demonstrate that individuals can discriminate between the glandular secretions of other owl monkeys based on pedigree relatedness and female fecundity, but that they can detect the odor of other owl monkeys when secretions are deposited on a surface. Owl monkeys spent much more time investigating the odor of other owl monkeys, and seem to prefer this activity in comparison to a control odor. The odors in the trials simulate scent-marks, suggesting that owl monkeys can perceive, and identify information about the signaler, from scent-marks. This finding reinforces the notion that scent-marks serve an intra-specific communicative function. This study presents the results from the first behavioral bioassays conducted in *Aotus*, and these results demonstrate that captive owl monkeys can respond well to choice tests, suggesting this method can be a valuable for future research on other chemosignals.

Mate Choice and Inbreeding Preference

The study of mate choice in the wild owl monkey population has revealed the first evidence of a preference for close inbreeding in a wild non-human primate population. Here, genetic estimates of relatedness between male-female pairs in the wild population of owl monkeys showed that some pairs are close kin. The frequency of these close kin pairings and the mean estimate of relatedness between pairs were much greater than expected if mating were random. This observation strongly suggests that individuals were not mating randomly nor avoiding inbreeding, instead pairing with close kin more frequently than expected. By contrast, numerous other studies of mating in wild primate populations have shown evidence that individuals avoid mating with close kin (Huchard et al., 2013, 2017; Wikberg et al., 2017).

The implications of mating between close kin in the wild owl monkey population, and the reason why some individuals choose to partner with close kin, are not clear at this point. It is possible that inbreeding depression is weak or non-existent among owl monkeys. In this case, the deleterious effects typically associated with close inbreeding would not be present to impose costs on inbreeding. Individuals who do pair with close kin may experience greater fitness, through inclusive fitness benefits, by caring for offspring that share more genetic material with them than if offspring were produced through outbreeding. It is also possible that there are deleterious effects associated with close inbreeding, but the inbreeding threshold is lower. Kokko and Ots (2006) developed a model outlining three criteria that lower the inbreeding threshold, and owl monkeys seem to fulfill these criteria: (1) both sexes invest heavily in infant care; (2) owl monkeys

infrequently encounter potential mates; and (3) reproduction is likely more similar to sequential than simultaneous mating.

Chemosignals of Kinship

The behavioral bioassays results support the hypothesis that olfactory cues are one potential mechanism for kin discrimination and kin preference in owl monkeys. These bioassays were designed to evaluate whether individuals can detect differences between other individuals using olfactory cues alone. When presented with glandular secretions from close kin and non-kin, owl monkeys spent more time investigating the secretions collected from close kin individuals. It is possible these putative chemosignals associated with relatedness are used in mate choice.

Chemosignals of Female Fecundity

In this study, I have found the first evidence to suggest there is a signal of female fecundity encoded in the glandular secretions of female owl monkeys. During the behavioral bioassays, males exhibited a strong preference for glandular secretions of fecund more than of less fecund females. This result strongly suggests that males have the ability to detect fecundity from olfactory cues alone, although the sample size in this particular study was small (n=6 trials).

Yet, the evidence is less compelling when looking at sexual behavior observed by breeding pairs. Among captive owl monkeys, males and females copulated slightly more often before ovulation, when a female is more fecund, than after ovulation, when there is no chance of conception. Still, the evidence in support of this interpretation was not

robust, and copulations occurred frequently throughout the study. Among wild pairs, copulations occurred much less frequently and mainly around ovulation. Even so, most of the observed copulations occurred after the female had ovulated, although this difference may be due, in part, to sampling bias. Overall, in both cases, mating pairs did not show substantially greater copulations when a female was most fecund.

Given these results, it is possible that the chemosignals are present, but not accurate, and may persist beyond ovulation. It may also be the case that olfactory and sexual behaviors serve additional purposes beyond, or instead of, coordinating reproduction. Specifically, they may play an integral role in establishing the bond shared between males and females, as it does in socially monogamous prairie voles (Williams et al., 1992; Curtis et al., 2001). Olfactory signaling and sexual behavior may play a greater role in forming or maintaining a pair bond than in coordinating reproduction.

Overall, the patterns of discrimination of fecund odors and poorly timed copulations noted in this study seem contradictory, yet may not differ much from sexual behavior observed in humans. Men discriminate between the odor of women based on ovulatory phase (Singh and Bronstad, 2001; Havlíček et al., 2006; Gildersleeve et al., 2012), although, within couples, there does not seem to be evidence that reproduction is timed for conception (Brewis and Meyer, 2005).

Sexual Dimorphism, Sexual Selection, and Olfactory Communication

Across non-human primates, there is substantial evidence that olfactory traits have been influenced by sexual selection (Heymann, 2003a; Snowdon, 2004; Drea, 2015; Setchell, 2016). Following Snowdon's (2004) criteria to identify sexually selected traits, I

evaluated the degree of sexual dimorphism and intra-sexual variation in olfactory traits as a first step to begin to evaluate whether olfactory traits were sexually selected. Owl monkeys do show some degree of dimorphism and substantial intra-sexual variation chemically, physically in the subcaudal gland size and the color and amount of perianal oil, and behaviorally in the frequency of genital inspections. These relatively low levels of sexual dimorphism related to chemical communication, compared to other non-human primates, may be associated with mating competition, which is likely similar between the sexes in owl monkeys. Evidence for signals of female fecundity also seem fulfill Snowdon's third and fourth criteria that individuals discriminate between variants of the trait and show preference for a particular variant related to reproduction. Males are able to discriminate between the odor of a female when she is more or less fecund, and preferentially attend to more fecund odor. This behavior strengthens the case that chemical communication in owl monkeys has been influenced by sexual selection.

Interestingly, the dimorphism observed in owl monkeys is much less than the degree of dimorphism of olfactory traits found in other non-human primates that are typically considered "monomorphic", including tamarins (Heymann, 2003b) and sifakas (Schilling, 1979; Lewis, 2005; Pochron et al., 2005). These differences in patterns of dimorphism of olfactory traits may offer an interesting comparative perspective with which to analyze sexual dimorphism in humans. Variation in reproduction and mating patterns across primates may be related to the presence of sexual selection in chemical communication, and it may be possible to extrapolate how human mating behavior may have influenced sexual dimorphism of olfactory traits, as well.

Integrating Captive and Field Research

Throughout much of this dissertation, I have been able to combine data collected from wild and captive populations to address my hypotheses and research questions. This approach has been effective and informative, and resulted in a more robust analysis of chemical communication in owl monkeys than could be accomplished with a study of captive or wild individuals alone. Only through field research can we learn about the adaptive value of putative signals, while the mechanisms by which male and female owl monkeys regulate these signals will never be fully understood through observational research alone. For example, in captivity, owl monkeys seem to have the ability to discriminate between individuals based on relatedness. Yet, only by examining the demographic and genetic data from wild owl monkey pairs is it possible to suggest that the potential adaptive value of kin discrimination is to preferentially mate with closely related individuals.

Combining approaches also provides the opportunity to contrast similar types of data collected in each different environment, and better understand whether similar mechanisms of olfactory signaling operate across the *Aotus* genus. For example, chemosignals of sex were apparent in the glandular secretions of both captive and wild owl monkeys. In addition, in the wild population, signals associated with age category were just as strong as sex, yet there was no obvious signal of age in the captive population. This finding is most likely due to differences in reproductive maturity and social housing between the two populations. The ability to compare olfactory responses across environmental contexts, in this case at least, enriches our understanding of the data and highlights the benefits of integrating research on captive and wild populations.

Conclusion and Future Directions

As shown in this dissertation, there is accumulating evidence indicating that chemical communication is an integral aspect of owl monkey behavior and social relationships. This research offers a valuable perspective for understanding the evolution of olfactory signals in primates, as most primate olfactory research has focused on non-monogamous species such as mandrills, lemurs, and callitrichines, and because field research is relatively sparse (Heymann, 2006a). This project represents the first comprehensive study of chemical communication in owl monkeys, a monogamous pair-living primate with no evidence of extra-pair paternity. Their mating system differs greatly from other non-human primates that have been more extensively studied with regard to chemical communication, including mandrills, ring-tailed lemurs, sifakas, and callitrichids, all of which show more flexible mating patterns than owl monkeys. The improved understanding of owl monkey chemical communication broadens our knowledge of the way that chemical communication varies with social and mating patterns. This project further provides us with the basis for drawing comparisons in chemical, behavioral, and physical olfactory traits across primate species and mating systems, and may serve as an interesting model for comparisons to human olfactory communication.

This dissertation sets a strong foundation to pursue several avenues of future research. Evaluating the presence of putative chemosignals associated with genetics, including relatedness or the major histocompatability complex, could provide more informative assessment of how kinship or other genetic variables that may be perceived

ultimately influence mate choice. Behavioral bioassays could further be used to investigate the ability of owl monkeys to detect other putative chemosignals, including sex and age, and examine whether owl monkeys can recognize individuals, such as their current or former partners, or offspring. Exploring sexual dimorphism in other aspects of owl monkey anatomy, such as the olfactory bulb, would be a valuable comparison to human sex differences. Yes, another potentially interesting avenue for future work would be the analysis of nonvolatile compounds, which may be involved in signaling. Such compounds could play a critical role in owl monkey chemical communication since they do possess vomeronasal organs (Hunter et al., 1984) and often make contact when investigating odors. Finally, exploring whether olfactory traits are correlated with reproductive success would improve our understanding of the potential function and adaptive value of olfactory communication in owl monkeys, and further strengthen the evidence that sexual selection has influenced chemical communication in owl monkeys.

APPENDIX 1

Standard solutions for 21 individual compounds were prepared in ethanol to produce solutions of ~1000ppm, which were stored at 4C. Mixed solutions of up to six compounds were prepared in water resulting in mixtures of multiple compounds each at 50ppm.

To identify the compounds for peaks in the *Aotus* samples, we re-analyzed wild and captive Aotus samples. We fortified some samples with the mixture while others remained unfortified. We conducted our dynamic headspace analysis with gas chromatography-mass spectrometry following the methodology described in the manuscript, with the following changes to account for the H_2O and ethanol in the mixes: a) sweep time was reduced from 30 to 10min, b) included a 1min dry purge, and c) delayed start time of the mass spectrometer from 3 to 7min.

Using the retention times and mass spectra of the compounds in the mixtures to both the fortified and unfortified samples, we were able to confirm the identity of eight peaks (see Supplementary Table 1). Additionally, we were able to rule out 13 compounds that were considered as possible peak identities (Supplementary Table 1).

Tables

Table 1: List of the compounds we compared to the peaks in the *A. azarae* and *A. nancymaae* sample. Correctly identified peaks are in bold

Species	Compound	Peak	Correct ID
A. azarae	**1-Butanol (≥99%)**	**1392**	**Yes**
	6-Methyl-5-hepten-2-one (≥99%)	2204	No
	2,6-Diethyl-pyrazine (98%)	2713	No
	Linalool (≥99%)	**2977**	**Yes**
	1-Methyl piperidine (≥99%)	4892	No
	4-Methyl piperidine (96%)	4892	No
	4-Ethyl phenol (≥99%)	**4964**	**Yes**
A. nancymaae	**4-Heptanone (≥99%)**	**1053**	**Yes**
	o-Xylene (≥99%)	1085	No
	2-Heptanone (98%)	**1297**	**Yes**
	Limonene (97%)	**1349**	**Yes**
	2-Pentyl-furan (≥99%)	**1448**	**Yes**
	2-Nonanone (≥99%)	1865	No
	3-Nonanone (≥96%)	1865	No
	4-Nonanone (≥99%)	**1865**	**Yes**
	5-Nonanone (≥98%)	1865	No
	Dimethyl disulfide (≥99%)	2108	No
	Dimethyl trisulfide (≥98%)	2108	No
	3-Ethyl-2,4 pentanedione (≥98%)	2453	No
	2-Decanone (≥98%)	2453	No
	4-Decanone (≥97%)	2453	No
	Benzoic acid (≥99%)	2507	No
	Benzaldehyde (≥99%)	**2718**	**Yes**
	Citral/geranial (≥95%)	3197	No
	Naphthalene (≥99%)	3473	No

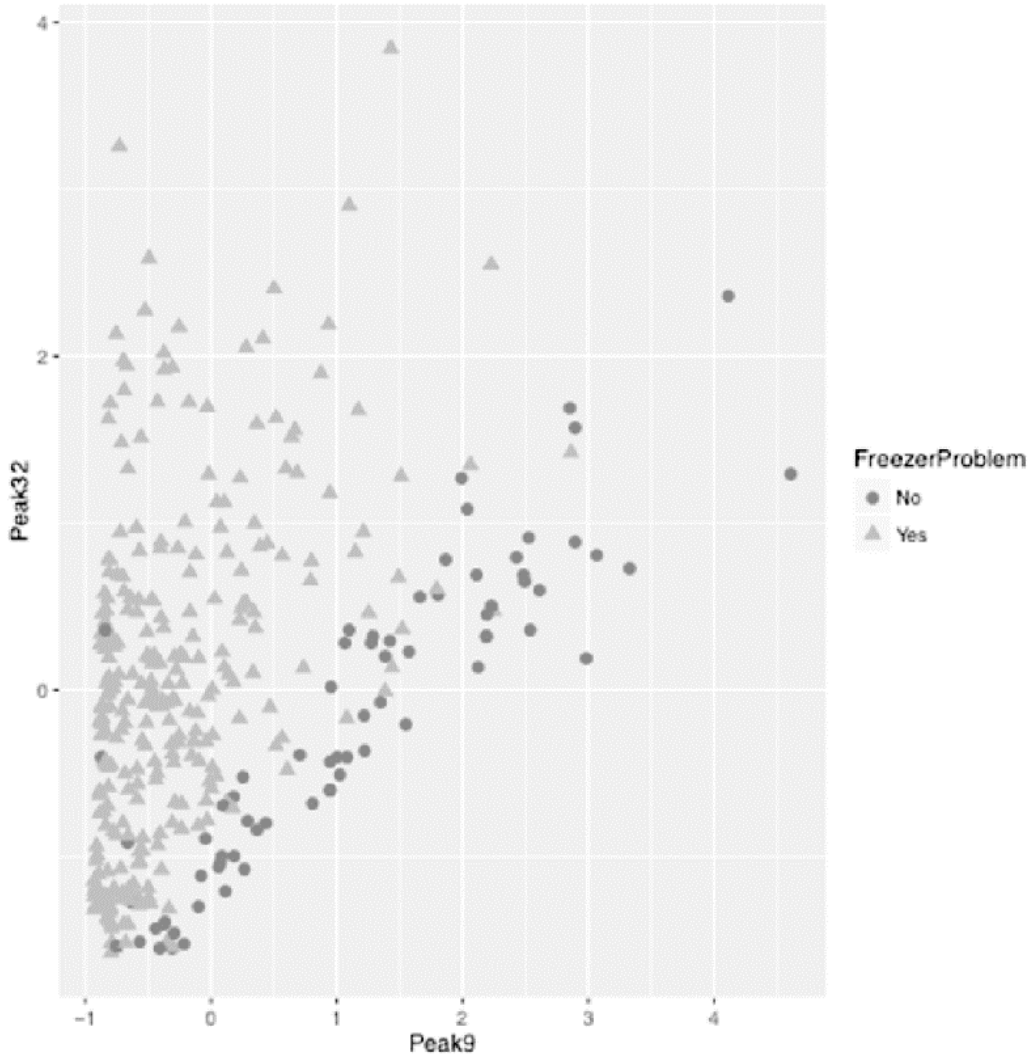
APPENDIX 2

The Owl Monkey Project has also collected 287 subcaudal and pectoral samples from 54 females and 56 males from 2001 to 2009 that were excluded from our analysis in Chapter 1. These samples experienced freezer failure in 2009, where the freezer stopped cooling and began heating its contents.

We compared the samples that underwent the freezer failure to those that did not, and found dramatic differences. The unaffected samples were found to have 36 more peaks (compounds), suggesting that the samples that experienced the freezer failure underwent a dramatic loss of volatile compounds. We also conducted a linear discriminant analysis, and found that we can distinguish between these two groups with 80% accuracy using only three variables (see Figure 1). Given these differences in the samples likely caused by the freezer failure, we choose to exclude these samples from analysis.

Figures

Figure 1: Square-root transformed and scaled relative peak values for the first two peaks in the LDA model to discriminate wild *A. azarae* by freezer status, including those samples that experienced a freezer failure ("Yes") and those that did not ("No")



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