IDENTIFYING CHEMICAL SIGNALS AND EXAMINING THEIR ROLE IN REPRODUCTION IN THE MANED WOLF (*CHRYSOCYON BRACHYURUS*)

by

Marieke Kester Jones
A Dissertation
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Date: _____ Summer 2017
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DEDICATION

This is dedicated to my family. To my parents, Adrian and Nancy, your scientific curiosity continues to be an inspiration to me. With your steadfast support and encouragement, I really can do anything. To my siblings, Julia and Arnie, thank you for your trips to Virginia and your countless hours on the phone. Keith, you really have embodied the definition of a partner throughout my PhD, taking the challenges and victories along with me, *pari passu*. Finally, without the comedic antics of my fur children, Jack and Aubrey, my writing environm150ent would have been much more boring, albeit cleaner and quieter.

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I first wish to thank my committee members. The four of you have truly made me the researcher and teacher I am today. I am deeply appreciative of Dr. Nucharin Songsasen for embarking on this journey with me since the very beginning when I started as her intern in 2009. Ever since, Nuch has supported me not only by providing sound scientific advice, but also through her insightful counseling and true mentorship all along the road to this dissertation. I am especially thankful for Nuch's persistent belief in me, trusting that I was making progress while I worked miles away to complete my coursework and chemistry lab work. Thank you Momma Duck! Dr. Larry Rockwood has made this whole ride possible. When Nuch and I first met with him back in 2010, I think he was as excited as I was that I would be teaching biostatistics. That teaching position quickly became one of the most fulfilling, satisfying aspects of my PhD. Thank you Dr. Rockwood for trusting me with ever-increasing levels of teaching responsibilities; designing the distance learning and honors courses were some of the best professional development opportunities I had during my tenure here. Dr. Rockwood unfailingly found funding when there seemed to be none around and always added humor to my days on the Fairfax campus. Dr. Elizabeth Freeman is the best life coach a student could ask for. I could always depend on her to help me see the light through whatever academic or scientific challenge I faced. She always found the time to provide an incredibly detailed, thorough review of my work, which made me a better scientist and writer. Thank you Elizabeth. Finally, I am grateful to have been advised by Dr. Thomas Huff. Without the instrumentation in the SRIF lab, substantial SRIF lab funding, and Tom to guide me, my research would have only been aspirational. Tom has always been passionate about my research and worked tirelessly (including many weekends!) to ensure that the instruments were functioning properly. Along the way, he taught me everything I know about how these machines function and about all the weird and wonderful ways to use mass spec.

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LIST OF ABBREVIATIONS AND/OR SYMBOLS

Volatile Organic Compound ..... VOC
Solid Phase Microextraction ..... SPME
Gas Chromatography-Mass Spectrometry ..... GC-MS
Headspace ..... HS
Maned Wolf Species Survival Plan ..... MWSSP
Gonadotropin Releasing Hormone ..... GnRH
Follicle Stimulating Hormone ..... FSH
Luteinizing Hormone ..... LH
Ovulation Induction Factor ..... OIF
Retention Time ..... RT
Institutional Animal Care and Use Committee ..... IACUC
Studbook Number ..... SB#
Mass to Charge Ratio ..... m/z
National Institute of Standards and Technology ..... NIST
Fold Change ..... FC
Principal Components Analysis ..... PCA
Partial Least Squares Discriminant Analysis ..... PLS-DA
Random Forests ..... RF
Standard Deviation ..... SD
Standard Error of the Mean ..... SEM
Chemical Abstracts Service Registry Number ..... CAS No.
International Union for Conservation of Nature ..... IUCN
Variable Influence on Projection ..... VIP
Out of Bag Error ..... OOB
Smithsonian Conservation Biology Institute ..... SCBI
Analysis of Variance ..... ANOVA
Fecal Estrogen Metabolites ..... FEM
Fecal Progestagen Metabolites ..... FPM
Fecal Androgen Metabolites ..... FAM
Fecal Cortisol Metabolites ..... FCM

ABSTRACT

IDENTIFYING CHEMICAL SIGNALS AND EXAMINING THEIR ROLE IN REPRODUCTION IN THE MANED WOLF (*CHRYSOCYON BRACHYURUS*)

Marieke Kester Jones, PhD

George Mason University, 2017

Dissertation Director: Dr. Larry Rockwood

The maned wolf (*Chrysocyon brachyurus*) is a Neotropical canid species that exhibits a rare reproductive mechanism, induced ovulation. Unlike most other canids, the maned wolf is solitary, so induced ovulation is an important adaptation to ensure successful reproduction. Though the mechanism of ovulation induction remains unknown, it is suspected to be a urinary chemical signal. This dissertation has three main objectives: (1) Identify volatile organic compounds (VOCs) that are consistently found in maned wolf urine; (2) Examine differences in VOCs according to sex and reproductive status to distinguish putative semiochemicals; and (3) Investigate the behavioral and hormonal responses of female maned wolves to male urine stimuli. Monthly urine samples were collected from 11 maned wolves (five males, six females) in 2013 and weekly samples were collected from 13 maned wolves (six males, one castrated male, six females) in 2014. Urinary VOCs were extracted using headspace solid-phase microextraction and gas

chromatography-mass spectrometry. The VOCs found in highest abundance and ubiquitously across samples were pyrazines, likely responsible for the pungent smell of maned wolf urine, and hemiterpenoid alcohols. The VOCs that contributed the most to classification as a male or female were 3-iodo-E-2-octenoic acid, 10-methyl-2-oxecanone, an unidentified compound, 1,1'-thiobis-cyclopentane, and 2-nonen-4-one. Fifty VOCs differed between intact males and a castrated male. The behavioral study assessed whether presenting male urine to females could prompt ovarian activity and behavioral interest without the physical presence of a male. Secondarily, this study investigated the effect of group housing females on reproductive hormones and behaviors. Three co-housed females were exposed to male urine for thirty days while four females housed as two sister pairs were controls and were not exposed. Daily behavioral observations were conducted on the treatment females pre-, during, and post-exposure. Fecal samples from all seven individuals were collected to assess metabolites of gonadal hormones. While none of the seven females ovulated, treatment females showed significantly higher estrogen metabolite concentrations than controls and behavioral interest was highest when urine was present. In two of the three groups, one female had higher reproductive hormone concentrations than conspecifics. Co-housed females engaged in agonistic encounters on average 8.5 times per hour. The female with the highest reproductive hormone levels was the dam, and she was the only individual to scent mark. This dissertation provided the most comprehensive analysis of maned wolf urinary VOCs to date and identifies several putative semiochemicals. The behavioral evidence supports the presence of urinary semiochemicals, and offered the first evidence

that reproductive suppression may occur when co-housing females. Future studies to bioassay the putative semiochemicals and to systematically study reproductive suppression are recommended to further elucidate reproductive mechanisms in the maned wolf.

CHAPTER 1: LITERATURE REVIEW

The maned wolf

The maned wolf (*Chrysocyon brachyurus*) is a unique member of the Canidae family, endemic to the tropical grassland habitats of South America (Dietz 1985). *Chrysocyon* is a monotypic genus thought to have developed first in North America followed by migration during the Great American Interchange (around three million years ago) to South America via the newly created isthmus (Macdonald and Sillero-Zubiri 2004; Sillero-Zubiri 2014). Based on mitochondrial DNA analyses, it appears that *Chrysocyon* and its most closely related genus, *Speothos*, must have diverged before the migration to South America, resulting in three separate canid colonizations of South America, one for *Chrysocyon*, one for the *Speothos,* and one for the rest of the South American canid lineages (*Lycalopex*, *Cerdocyon,* and *Atelocynus*) (Macdonald and Sillero-Zubiri 2004; Sillero-Zubiri 2014). *Chrysocyon* and its sister taxon, *Speothos*, are classified along with *Lycaon* as basal to the wolf-like canid clade, which is comprised of the *Canis* and *Cuon* genera (Sillero-Zubiri 2014).

Standing 90 cm at the shoulder, the maned wolf is the tallest of the canids, though at an average weight of 23 kg, it is certainly not the heaviest (Dietz 1985). The species is named for the long black hairs across its neck and shoulders that contrast starkly with its overall red-golden color. Long legs, an unusual lateral gait, and large erect ears are adaptations that help the species thrive in the tall grassland vegetation dominating its

native landscape (Queirolo et al. 2011). Maned wolves prefer open-canopy vegetation comprising tall grasses, shrubs, and occasional woodlands (Jácomo et al. 2009; Queirolo et al. 2011). Typically, large canids (>21 kg) live in groups to optimize prey capture, metabolic efficiency, and reproductive success (Macdonald and Sillero-Zubiri 2004). However, due to resource availability constraints, the maned wolf has evolved an atypical diet and social system. Unlike most other large canids whose diets consist largely of animal prey, the maned wolf diet is around 50% small mammals, birds, and reptiles, with the other 50% comprised of vegetative matter, notably the lobeira fruit (*Solanum lycocarpum*) (Motta-Junior et al. 1996; Aragona and Setz 2001; Jácomo et al. 2004; Emmons 2012; Motta-Junior et al. 2014).

Without the need for a pack to optimize prey capture, the maned wolf has evolved a solitary social system with seasonal, facultative monogamy (Macdonald and Sillero-Zubiri 2004). Monogamous pairs defend a shared territory (20 – 90 km^2) but meet up only briefly during the breeding season (Kleiman 1972; Dietz 1984; Emmons 2012). As of yet, there are no firm data on the age of reproductive maturity (Songsasen and Rodden 2010). However, data from the International Studbook suggest that first copulations can occur between 1 – 2 years of age in both sexes (Rodden et al. 2007). Prime reproductive years are considered ages 3 – 8 (Songsasen et al. 2006; Rodden et al. 2007). Reproductive senescence is typically reached around age 12 in both sexes (Rodden et al. 2007; Songsasen and Rodden 2010).

While the diet and social system of the maned wolf is atypical of Canidae, reproduction in the maned wolf is, in most ways, characteristic of other wild canids. Like

most canids, the maned wolf is a seasonal monoestrous breeder meaning that females cycle just once per year during breeding season (Asa and Valdespino 1998; Sillero-Zubiri et al. 2004). As an adaptation to monoestrous females, males produce sperm only during breeding season, and remain azoospermic for the remainder of the year (Wildt et al. 2010; Johnson et al. 2014b). This is similar to seasonal sperm production in other large canids (Johnston et al. 2007; Minter and DeLiberto 2008).

The onset of breeding season for the maned wolf appears to be a response to a decreasing light cycle (Maia and Gouveia 2002; Rodden et al. 2007; Valdespino 2007). For this species, breeding takes place in the northern hemisphere from September through February (with a mean date of November) and from March through June in South America (Kleiman 1972; Rodden et al. 1996; Maia and Gouveia 2002; Rodden et al. 2007; Songsasen et al. 2014). The maned wolf gestation period is approximately 65 days (Dietz 1985). On average, litter sizes are smaller (1 – 7 pups, mean = 2.6) than most other canids (Maia and Gouveia 2002; Songsasen et al. 2014).

Intriguingly, the maned wolf seems to be an induced ovulator, meaning that females ovulate only in the presence of a male (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). Although not all canid species have been investigated for this trait, as of yet the only other presumed induced ovulating canid is the Channel Island fox (*Urocyon littoralis*) (Asa et al. 2007). The domestic dog (*Canis familiaris*) (Concannon et al. 2009; Concannon 2011), gray wolf (*Canis lupus*) (Seal et al. 1979; Asa et al. 2006), coyote (Carlson and Gese 2008), African wild dog (Monfort et al. 1997; Van der Weyde et al. 2015), bush dog (*Speothos venaticus*) (DeMatteo et al. 2006), red fox (*Vulpes*

vulpes) (Mondain-Monval et al. 1977; Maurel et al. 1984), and arctic fox (*Alopex lagopus*) (Möller 1973) are known to be spontaneous ovulators.

Female maned wolves not paired with a male show baseline progestagen levels through the entire breeding season, demonstrating a lack of ovulation (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). In 2004, a pair that had been separated for the duration of the normal breeding season to prevent pregnancy copulated shortly after being reintroduced in April and gave birth more than two months outside the typical breeding and birthing season (Rodden et al. 2007). This suggests that the presence of a male strongly influences the timing of ovulation. In 2009, a female housed singly but sharing a fence line with a male ovulated. In this instance the female had visual access to the male as well as the ability to contact his urine scent marks deposited on the shared fence line (Songsasen, pers. comm.). In the same reproductive season, several other females in visual, but not direct olfactory, contact with males failed to ovulate, suggesting an olfactory mechanism underlying this phenomenon.

Conservation of the maned wolf

The species is currently listed as "Near Threatened", with an estimated wild population of around 20,000 (Paula and DeMatteo 2015). Brazil is known to hold the vast majority of the wild population with less than 1000 individuals each in Argentina, Bolivia, and Paraguay (Songsasen and Rodden 2010; Queirolo et al. 2011; Paula and DeMatteo 2015). Major threats to conservation of the species in the wild include conversion of habitat into agricultural land (Vynne 2014; Paula and DeMatteo 2015), road mortality (Motta-Junior et al. 1996), conflicts with humans (Sillero-Zubiri et al.

2004; Vynne 2010), and the spread of domestic dog diseases (Motta-Junior et al. 1996; Deem and Emmons 2005; May-Junior et al. 2009; Lacerda et al. 2009; Curi et al. 2010; Furtado et al. 2016). Climate change is forecasted to exacerbate the worst of these threats, with predictions of habitat loss of at least one third of the current maned wolf distribution (Torres et al. 2013). Only 39% of the species' current distribution is considered stable until 2050 (Torres et al. 2013).

Ex situ conservation

Despite conservation efforts in the wild, preservation of a species often requires maintaining healthy populations in captivity for several purposes: to supplement an ailing wild population, to provide a safeguard against unexpected catastrophes leading to extinctions of wild populations, to educate the public about the species, and finally, to provide opportunities to research the basic biology of the species in order to improve conservation efforts (Songsasen and Rodden 2010; Jewgenow and Songsasen 2014). Captive breeding programs have been used to successfully nurse populations of endangered species back from the brink of extinction including *inter alia* the black-footed ferret (*Mustela nigripes*), golden lion tamarin (*Leontopithecus rosalia*), red wolf (*Canis rufus*), California condor (*Gymnogyps californianus*), scimitar-horned oryx (*Oryx dammah*), giant panda (*Ailuropoda melanoleuca*), Iberian lynx (*Lynx pardinus*), and peregrine falcon (*Falco peregrinus*) (Seddon et al. 2007; Wildt et al. 2010; Jewgenow and Songsasen 2014).

In 2016 there were 78 maned wolves distributed across 32 institutions in North America (Songsasen, pers. comm.), down from 88 in 2014 (Songsasen et al. 2014). All

32 institutions participate in the Maned Wolf Species Survival Plan (MWSSP) in an effort to maximize genetic diversity. As of 2012 the *ex situ* population had maintained a genetic diversity of 92.7% (Rodden 2012), declining to 91.06% in 2016. The MWSSP has set a goal of maintaining at least 90% genetic diversity through the next 100 years (Rodden 2012). However, extrapolating the recent poor reproductive success into the future, this population is estimated to maintain 90% genetic diversity for only the next five years, and decline to 35.6% genetic diversity over 100 years (Rodden 2012). Therefore, the MWSSP has placed a high priority on research focusing on the reproductive biology of the maned wolf to enhance genetic management of the population.

Knowledge about reproduction is integral to species viability *in situ* and the success of captive breeding programs (Wildt and Wemmer 1999; Pukazhenthi and Wildt 2004; Comizzoli et al. 2009; Wildt et al. 2010). Over multiple decades, researchers have found that there are almost as many mechanistic differences in carnivore reproduction as there are species (Wildt et al. 2010; Jewgenow and Songsasen 2014). For the world's 55,000 vertebrate species, extensive knowledge of reproductive mechanisms exists for a mere 0.25%, and only ~2% of mammals have been well studied (Wildt et al. 2003). Thus, improving knowledge of reproduction and identifying novel reproductive mechanisms can help to enhance populations and conservation for the given species, but can also lead to practical applications in other wildlife species, and possibly in humans as well (Wildt et al. 2010). As a result, investigating species-specific aspects of wildlife reproduction,

especially focusing on defining novel and unique reproductive mechanisms, is the highest priority of wildlife research today (Pukazhenthi and Wildt 2004; Comizzoli et al. 2009).

Reproductive endocrinology

The regulatory dynamics of the reproductive cycle are highly complex, requiring a concert of hormones generated in the hypothalamic-pituitary-gonadal axis (Figure 1). Hormones also interact with each other in intricate feedback loops to generate proper reproductive function. Specifically, gonadotropin releasing hormone (GnRH), originating in the hypothalamus, stimulates the release of two gonadotropic hormones from the anterior pituitary, follicle stimulating hormone (FSH) and luteinizing hormone (LH), molecules that are central to reproductive cyclicity (Senger 2003). FSH is responsible for stimulating the growth of follicles and for the production of estrogen (Senger 2003). Estrogen also serves in a positive feedback loop to up-regulate the production of GnRH. GnRH prompts the release of LH, which eventually leads to an LH surge, followed by an estrogen peak, and ovulation (Senger 2003).

Ovulation results in the formation of a corpus luteum (Senger 2003). The corpus luteum produces progesterone, which serves to down-regulate GnRH production halting the production of FSH and LH through the pregnant or non-pregnant luteal phase (Senger 2003; de Gier et al. 2006; Concannon 2011) (Figure 1). Luteolysis, the disintegration of the corpus luteum, is accomplished by oxytocin from the corpus luteum and, in most species, by prostaglandin $F_{2\alpha}$ release from the uterine endometrium (Senger 2003). It is thought that oxytocin stimulates prostaglandin $F_{2\alpha}$ production in the endometrium that

travels back to the ovary and results in a positive feedback loop prompting luteolysis and thus, a new follicular phase (Senger 2003).

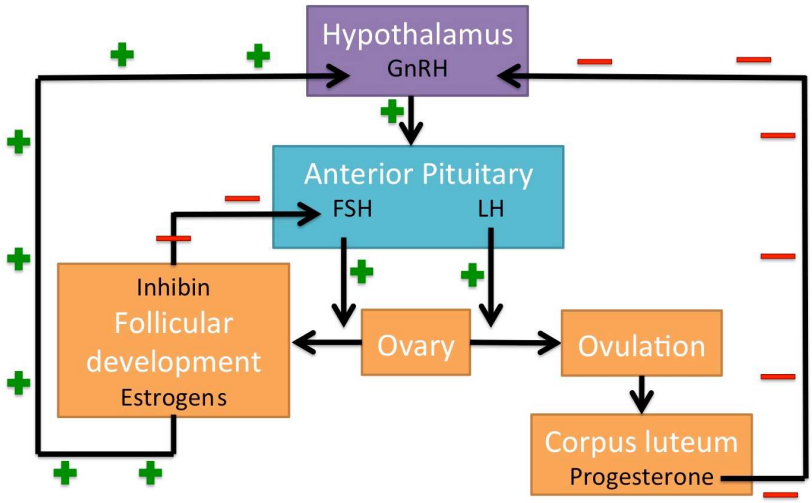


Figure 1. Simplified flow chart of the hypothalamic-pituitary-gonadal axis in the female showing the negative and positive feedback systems (based on Senger 2003).

In the domestic dog ovulation occurs two days after the LH surge and four days after the estrogen peak (de Gier et al. 2006; Concannon 2011). Canids differ from other taxa in the hormone ratios present during ovulation and fertilization. As opposed to most mammals, canid preovulatory follicles luteinize prior ovulation. This luteinized follicle secretes progesterone and therefore, egg maturation and fertilization occurs in an elevated progesterone environment, a significant departure from the hormones present during fertilization in other taxa (Wildt et al. 1979; Songsasen et al. 2006). A second difference between the typified mammalian endocrinology presented above and that of the canid is that in the domestic dog the uterus does not seem to play a role in luteolysis (Senger 2003; Concannon 2011). Although prostaglandin $F_{2\alpha}$ does have a luteolytic effect, it is

not secreted from the canid uterus at concentrations that would affect luteal function (Concannon 2011). Instead, dogs experience a slow luteal regression and the associated steady decline of progesterone over several weeks (Concannon 2011). This decline is then followed by a long period of ovarian inactivity called anestrus prior to the subsequent follicular phase (Concannon et al. 2009; Concannon 2011).

Maned wolf reproductive endocrinology

Non-invasive fecal hormone monitoring has been used with great success to assess reproductive hormones in the maned wolf (Wasser et al. 1995; Velloso et al. 1998; Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). Using this method, researchers have determined that the maned wolf follows patterns of gonadal hormone secretion similar to that of the domestic dog (de Gier et al. 2006; Concannon 2011) and to other wild canids (Monfort et al. 1997; Walker et al. 2002; DeMatteo et al. 2006; Asa et al. 2006; Asa et al. 2007).

There are four stages of the canid reproductive cycle: proestrus, estrus, diestrus, and anestrus. In the maned wolf, proestrus lasts up to 18 days and is characterized by vaginal swelling and secretions as well as a marked increase in courtship behaviors (Rodden et al. 1996; Velloso et al. 1998; Songsasen et al. 2006). Hormonally, proestrus is characterized by increasing levels of estrogen and progestagens (Figure 2) (Jewgenow and Songsasen 2014; Songsasen et al. 2014). Estrus is the period of reproductive receptivity and is so named due to the estrogenic peak during this period. Estrus lasts between 1 and 10 days, and is characterized by vulvar swelling, vaginal discharge, increased time spent in close proximity, frequent licking and sniffing of the mate's

anogenital region, and breeding attempts (Rodden et al. 1996; Songsasen et al. 2006; Rodden et al. 2007; Songsasen et al. 2014).

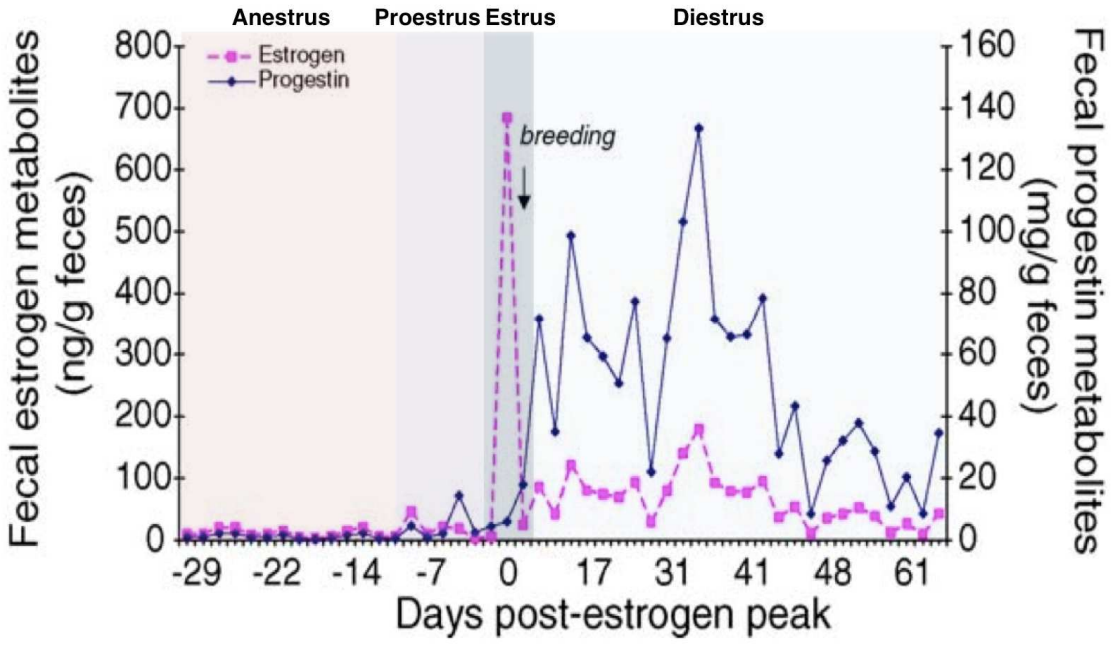


Figure 2. Longitudinal profiles of fecal estrogen and progestin metabolites for a pregnant maned wolf from 14 days before the estrogen peak to two weeks before parturition (from Songsasen et al. 2006).

Ovulation is thought to occur several days following the estrogen peak, during a time of rising progestagen concentrations, as in the domestic dog (Wasser et al. 1995; Velloso et al. 1998; Valdespino et al. 2002; Songsasen et al. 2006; Concannon et al. 2009; Van den Berghe et al. 2012). Copulation begins 2 – 6 days after the estrogen peak, and can last up to nine days after the peak (Songsasen et al. 2006). Successful breeding requires a copulatory tie lasting up to several minutes (Songsasen et al. 2014), a feature common across Canidae. On the day of successful copulation, estrogen levels decrease back to baseline (Songsasen et al. 2014).

Progestagen concentrations increase beginning in late proestrus and continue to increase through the estrus period into diestrus, peaking around 16 days after the estrogen surge (Figure 2) (Songsasen et al. 2006). Regardless of pregnancy status, females who ovulate show a ~65 day rise in progestagens following ovulation (Songsasen et al. 2006). This pattern of progestagen secretion is typical of domestic dogs and wild canids (Monfort et al. 1997; Walker et al. 2002; DeMatteo et al. 2006; de Gier et al. 2006; Asa et al. 2006; Asa et al. 2007; Concannon 2011). Changes in a female maned wolf's appearance during gestation are minimal, making differentiation between pregnant and non-pregnant females difficult (Rodden et al. 2007; Reiter 2012). However, using non-invasive endocrinology, researchers can detect significant differences between the fecal gonadal hormones of pregnant and non-pregnant luteal phases of this species. Pregnant maned wolves show higher levels of estrogen compared to non-pregnant females, but this difference is only significant after the fourth week of gestation (Wasser et al. 1995; Velloso et al. 1998; Reiter 2012). Additionally, non-pregnant females excrete lower levels of progestagens than pregnant females (Wasser et al. 1995; Velloso et al. 1998; Songsasen et al. 2006; Reiter 2012). Following parturition or a non-pregnant luteal phase and the regression of the corpus luteum, females enter an obligate anestrus phase for the remainder of the year until the next breeding season proestrus begins (Rodden et al. 2007).

Male maned wolves show high levels of testosterone through the breeding season, with the highest levels recorded for individuals in North America in December and January and the lowest in April (Songsasen et al. 2006). While most canids that have

been studied produce at least some sperm throughout the breeding season (Green et al. 1984; Goodrowe et al. 1998; Johnston et al. 2007; Jewgenow and Songsasen 2014), maned wolves only produce sperm for 6 – 8 weeks, and spermatogenesis declines precipitously immediately following successful breeding (Songsasen et al. 2006; Songsasen and Rodden 2010; Jewgenow and Songsasen 2014; Johnson et al. 2014b).

Induced ovulation

There are two general patterns of ovulation in mammals: spontaneous and induced ovulation. Spontaneous ovulation is characterized by reproductive hormone cycling with ovulation brought on by the positive feedback of estradiol on the pulsatile release of GnRH and LH (Bakker and Baum 2000; Adams and Ratto 2013). Induced ovulation occurs in females that release ova in response to an LH surge through neural activation via copulatory cues (Larivière and Ferguson 2003). In induced-ovulating females, the feedback loops responsible for ovulation in spontaneous ovulators are likely absent or dramatically reduced (Bakker and Baum 2000). Induced ovulating mammals are present in several orders (Table 1).

Induced ovulation ensures optimal gamete synchronization, and thus, a high probability of fertilization, factors especially important for solitary species (Conaway 1971). In North American carnivores, induced ovulation is more prevalent for seasonally breeding species (Larivière and Ferguson 2003). Induced ovulation tends to be more prevalent for species with large home ranges and long estrous cycles, presumably as a mechanism to ensure successful reproduction (Larivière and Ferguson 2003).

Table 1. Induced ovulating mammals and the mechanism of ovulation if known.

Taxon	Common name	Family	Order	Mechanism	Reference
Camelus bactrianus	Bactrian camel	Camelidae	Artiodactyla	Ovulation-inducing factor (OIF) in seminal fluid	Pan et al. 2001; Larivière and Ferguson 2002; Senger 2003; Adams and Ratto 2013
Camelus dromedarius	Dromedary	Camelidae	Artiodactyla	OIF	Nagy et al. 2005
Lama glama	Llama	Camelidae	Artiodactyla	OIF	Adams et al. 2005
Vicugna pacos	Alpaca	Camelidae	Artiodactyla	OIF	Adams et al. 2005
Chrysocyon brachyurus	Maned wolf	Canidae	Carnivora	Olfactory?	Reiter 2012; Johnson et al. 2014a
Urocyon littoralis	Channel Islands fox	Canidae	Carnivora		Asa et al. 2007
Panthera tigris	Tiger	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Felis concolor	Puma	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Panthera uncia	Snow leopard	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Acinonyx jubatus	Cheetah	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Leopardus tigrinus	Tigrina	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Leopardus pardalis	Ocelot	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Lynx spp.	Lynx	Felidae	Carnivora	Multiple Copulations	Bakker and Baum 2000; Brown 2011
Mephitis mephitis	Striped skunk	Mephitidae	Carnivora		Larivière and Ferguson 2002
Spilogale putorius	Spotted skunk	Mephitidae	Carnivora	Copulation	Mead 1966
Enhydra lutris	Sea otter	Mustelidae	Carnivora	Copulation	Mead 1966; Amstislavsky and Ternovskaya 2000

Table 1 continued. Induced ovulating mammals and the mechanism of ovulation if known.

Taxon	Common name	Family	Order	Mechanism	Reference
Galictis cuja	Lesser grisón	Mustelidae	Carnivora	Copulation	Amstislavsky and Ternovskaya 2000
Gulo gulo	Wolverine	Mustelidae	Carnivora	Copulation	Mead 1966; Amstislavsky and Ternovskaya 2000; Larivière and Ferguson 2003
Lontra canadensis	North American river otter	Mustelidae	Carnivora		Mead 1966; Amstislavsky and Ternovskaya 2000
Martes americana	American marten	Mustelidae	Carnivora		Mead 1966; Larivière and Ferguson 2002
Martes pennanti	Fisher	Mustelidae	Carnivora		Mead 1966; Frost 1994; Larivière and Ferguson 2002
Mustela erminea	Stoat	Mustelidae	Carnivora		Larivière and Ferguson 2002
Mustela eversmanii	Siberian polecat	Mustelidae	Carnivora	Copulation	Amstislavsky and Ternovskaya 2000
Mustela frenata	Long-tailed weasel	Mustelidae	Carnivora	Copulation	Mead 1966; Amstislavsky and Ternovskaya
Mustela nigripes	Black-footed ferret	Mustelidae	Carnivora	Copulation	Amstislavsky and Ternovskaya 2000
Mustela nivalis	Least weasel	Mustelidae	Carnivora		Mead 1966; Larivière and Ferguson 2002
Mustela putorius	Ferret	Mustelidae	Carnivora	Copulation	Mead 1966; Villars et al. 1990
Neovison vison	American mink	Mustelidae	Carnivora		Mead 1966
Taxidea taxus	European badger	Mustelidae	Carnivora		Mead 1966; Larivière and Ferguson 2002
Procyon lotor	Raccoon	Procyonidae	Carnivora		Larivière and Ferguson 2002
Ursus americanus	Black bear	Ursidae	Carnivora		Wimsatt 1963; Palmer et al. 1988; Boone et al. 2004
Ursus arctos	Brown bear	Ursidae	Carnivora		Herrero and Hamer 1977; Larivière and Ferguson 2002
Ursus maritimus	Polar bear	Ursidae	Carnivora		Larivière and Ferguson 2002
Delphinapterus leucas	Beluga	Monodontidae	Cetacea		Steinman et al. 2012

Table 1 continued. Induced ovulating mammals and the mechanism of ovulation if known.

Taxon	Common name	Family	Order	Mechanism	Reference
Monodelphis domestica	Gray short-tailed opossum	Didelphidae	Dildelphimorphia	Olfactory	Baggott et al. 1987; Hinds et al. 1992; Stonerook and Harder 1992; Jackson and Harder 1996; Harder and Jackson 2003; Vitazka et al. 2009
Bettongia penicillata	Brush-tailed bettong	Potoroidae	Diprotodontia		Hinds and Smith 1992
Oryctolagus cuniculus	European rabbit	Leporidae	Lagomorpha	Copulation	Conaway 1971; Sawyer and Radford 1978; Ramirez and Soufi 1994; Melo and González-Mariscal 2010
Cryptomys hottentotus	Common mole-rat	Bathyergidae	Rodentia	Copulation	Malherbe et al. 2004; Jackson and Bennett 2005
Georychus capensis	Cape mole-rat	Bathyergidae	Rodentia	Copulation	van Sandwyk and Bennett 2005
Microtus spp.	Voles	Cricetidae	Rodentia	Copulation	Breed 1972; Carter et al. 1980; Lepri & Vandenbergh 1986; Lepri & Wysocki 1987; Bakker & Baum 2000
Microtus ochrogastor	Prairie vole	Cricetidae	Rodentia	Olfactory	Richmond and Conaway 1969
	Squirrel family	Sciuridae	Rodentia		Conaway 1971
Tachyoryctes splendens	East African root rat	Spalacidae	Rodentia		Katandukila & Bennett 2016

One ovulation induction mechanism that has been well studied in rabbits and felids involves tactile stimulations of the cervix and vagina that stimulate sensory nerve channels triggering hypothalamic neurons that then cause the preovulatory surge of GnRH and LH (Bakker and Baum 2000; Senger 2003). While the exact mechanism of GnRH activation is still poorly understood, it is thought to include release of norepinephrine (Bakker and Baum 2000), neuropeptide Y (Bakker and Baum 2000), and protein neurohormone kisspeptin (Inoue et al. 2011).

Another known mechanism of ovulation induction is the ovulation-inducing factor (OIF) found in the seminal fluid of camelids (Table 1). Intriguingly, OIF from one species injected intramuscularly into other species[1] causes dose-dependent LH surges to occur, although the magnitude of the effect is species-dependent (Waberski et al. 1999; Pan et al. 2001; Senger 2003; Adams et al. 2005; Ratto et al. 2006; Bogle et al. 2012; Adams and Ratto 2013). Several of the recipient species are not induced ovulators suggesting that the OIF molecule is highly conserved across taxa as it plays a role in ovulation induction even in species that ovulate spontaneously. For example, although OIF from llama (*Llama glama*) seminal plasma does not induce ovulation in heifers (*Bos taurus*), it affects the follicular wave dynamics, hastening the regression of the current dominant follicle and prompting a new follicular wave (Adams and Ratto 2013).

Artificially inducing estrus and ovulation

For assisted reproductive techniques, it is advantageous to be able to manipulate the reproductive cycle to ensure that a recipient female is prepared to receive sperm or

[1] alpacas (*Vicugna pacos*), llamas, rabbits, mice (*Mus musculus*), horses (*Equus ferus caballus*), pigs (*Sus scrofa domesticus*), cows, guinea pigs (*Cavia porcellus*)

embryos or to facilitate oocyte collection for *in vitro* assisted reproductive techniques. This is accomplished via estrus or ovulation induction through the use of exogenous GnRH or gonadotropins. In canids, researchers have successfully induced estrus and/or ovulation in the domestic dog (Park et al. 2011; Walter et al. 2011; von Heimendahl and Miller 2012), gray wolf (Asa et al. 2006), coyote (Carlson and Gese 2009), and maned wolf (Johnson et al. 2014a).

In maned wolves a GnRH agonist is able to induce estrus and ovulation in paired females, but not in females housed singly (Johnson et al. 2014a). However, singleton females treated with the GnRH agonist followed by exogenous LH treatment are induced to ovulate successfully (Johnson et al. 2014a). Asa et al. (2006) used an exogenous GnRH implant to induce estrus in the gray wolf. Analysis of fecal hormone profiles indicates that the implant induced estrus in all females, but the timing of ovulation varies (Asa et al. 2006). In a study investigating the mutability of reproductive seasonality in the coyote, researchers are able to induce some hormonal and behavioral signs of reproduction outside of the normal breeding season using exogenous GnRH (Carlson and Gese 2009). However, it is not clear whether this procedure results in ovulation because the rise in progesterone is not prolonged for nine weeks as it is during a normal reproductive cycle.

Scent marking

Chemical signaling through scent-marking is central to social recognition, territory control, reproduction, and alarm signaling across Mammalia (Wyatt 2014). In fact, emerging evidence suggests that chemical signaling is the most pervasive form of

communication across all animal taxa (Wyatt 2014). Because olfactory communication does not rely on the presence of the signal sender, this type of signaling is persistent in the environment even after the sender has departed. For example, African wild dog scent marks continue to elicit strong behavioral responses from conspecific receivers up to ten weeks later (Parker 2010). This is because these marks contain high molecular weight compounds that resist degradation (Wyatt 2014). The substrate may even extend the life of chemical signals; for instance, highly polar compounds like short chain fatty acids could bind tightly to soil minerals, prolonging the scent signal's release (Parker 2010). This feature of scent-marking means that olfactory communication is likely especially important for nocturnal or solitary species that rarely come into direct contact with conspecifics (Hagey and MacDonald 2003; Delbarco-Trillo et al. 2011; Kean et al. 2011; Harris et al. 2012; Wyatt 2014).

Most olfactory communication research in canids has been conducted in social, group-living species. For these species, scent-marking communicates social status, territory maintenance, and reproduction (Dunbar 1977; Asa et al. 1984a; Hradecky 1985; Asa et al. 1990; Gese and Ruff 1997; Pal 2003; Wirant et al. 2007; Lisberg and Snowdon 2009; Parker 2010; Dehnhard 2011; Ausband et al. 2013; Berthoud et al. 2013; Jordan et al. 2013; Anhalt et al. 2014). In general, males urine-mark more frequently than females (Wells and Bekoff 1981; Hradecky 1985; Asa et al. 1990; Pal 2003), and dominant individuals scent-mark far more frequently than subordinates (Asa et al. 1984a; Asa et al. 1990; Gese and Ruff 1997; Parker 2010; Jordan et al. 2013).

African wild dogs respond at higher rates to urine compared to feces and urinate more frequently than they defecate (Parker 2010; Jordan et al. 2013). This species and the rest of Canidae assume specialized postures to urinate but not to defecate (Asa et al. 1984a; Wirant et al. 2007; Parker 2010; Jordan et al. 2013). Finally, urine scent-marking (but not defecation) increases in frequency during the breeding season for several canid species including the maned wolf (Hradecky 1985; Asa et al. 1990; Rodden et al. 1996; Gese and Ruff 1997; Pal 2003; Parker 2010; Apps et al. 2012; Jordan et al. 2013). Thus, urine is considered to be a more important source of scent signaling than feces for the Canidae (Gese and Ruff 1997; Parker 2010; Jordan et al. 2013).

In domestic dogs, the urine of estrous females is more attractive to males than that of non-estrous females (Dunbar 1977), suggesting that urine odor is closely associated with reproduction. Urine from intact males and females elicits longer investigation times than samples from gonadectomized individuals (Lisberg and Snowdon 2009), and intact males exhibit far more flehmen-like responses to conspecific urine than castrated males (Berthoud et al. 2013). Female domestic dogs in proestrus and estrus direct more of their urinations onto objects than females in other reproductive stages (Wirant et al. 2007), suggesting that urine scent-marks contain information about reproductive status. Additionally, males urinate more frequently in the vicinity of preferred individuals and preferred odor stimuli (Dunbar 1977). Together, these studies implicate the dependence of both urine marking and chemosensing behaviors on reproductive steroid hormone levels.

Like other canids, the maned wolf is highly territorial, marking its home range boundary with urine and feces deposited in conspicuous locations such as on top of termite mounds, ant mounds, next to shrubs or trees and along roads and rivers (Dietz 1984; Emmons 2012). Maned wolf feces and urine have a very distinctive pungent odor with scent-marks remaining detectable to humans for several weeks (Emmons 2012). Scent-marking rates do not differ between males and females (Songsasen et al. 2014), and are closely tied to territory maintenance; individuals who do not hold a territory do not scent-mark (Bestelmeyer 2000).

Scent marking also seems to play a role in reproduction for the maned wolf. For males, the frequency of urine scent-marking is highest during proestrus compared to other stages of the reproductive cycle, and is significantly higher in successful breeders when compared to unsuccessful breeders (Rodden et al. 1996). Similarly to males, successfully breeding females scent-mark significantly more than unsuccessfully reproducing females (Rodden et al. 1996). For females who attempt to breed but do not give birth, scent marking is highest during proestrus. However for females who successfully give birth, scent marking is highest during estrus (Rodden et al. 1996).

Semiochemicals in mammals

What is a pheromone?

One of the original definitions of a pheromone is a "substance secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behaviour or developmental process" (Karlson and Lüscher 1959, pp. 55). This definition seems to work well for

insects where responses are pre-programed and consistent over time, but for mammals, this definition of a pheromone becomes problematic (Brennan and Keverne 2004; Müller-Schwarze 2006; Doty 2010). In general, mammals are far more complex in the chemical mixtures they produce. Their responses to chemical secretions are also more complex than those of insects, often lacking the reflexive behavioral response previously viewed as a prerequisite of a pheromone (Brennan and Keverne 2004; Burger 2005; Müller-Schwarze 2006; Wyatt 2014). Instead, mammals adapt their behavioral responses to the message contained in the chemical signal. Thus, a more modern definition of "pheromone" allows for some variations in response behaviors, defining a modern mammalian pheromone as "a substance that is utilized for intra-specific communication even though it does not elicit apparent behaviour or endocrine changes" (Dehnhard 2011, pp. 55). Other authors prefer to use "chemical signal" or "semiochemical" to describe a mammalian pheromone (Burger 2005; Brennan and Kendrick 2006; Wyatt 2014), thus, I will use semiochemical throughout.

The understanding of semiochemicals in mammals lags far behind that of insect pheromones due to the complexity of the exocrine secretions of mammals (Burger 2005; Müller-Schwarze 2006; Wyatt 2014), making isolation and identification of the signaling compound(s) difficult. Glandular secretions in mammals contain a variety of chemical types including acids, alcohols, aldehydes, amines, aromatics, ketones, proteins, and sulfur-containing compounds (Burger 2005). In some cases, the bioactive signal may be encoded in the relative concentration of multiple compounds (of potentially different chemical compound classes) rather than simply by the presence or concentration of one

compound in particular (Novotny and Soini 2008; Wyatt 2014). Additionally, compounds identified as semiochemicals can be highly specialized and stereospecific, meaning that only one stereoisomer has bioactivity. This is the case for the semiochemicals of the house mouse and of the Asian elephant (*Elephas maximus*) (Novotny and Soini 2008).

Because of this complexity, sophisticated analytical chemistry methods coupled with complex multivariate statistical analyses become essential to derive meaning from copious amounts of data (Dixon et al. 2006; Novotny and Soini 2008). However, it should be noted that out of the 31 compounds identified as mammalian pheromones to date, 16 are a single compound and 15 are coded by presence/absence of a few compounds, so the possibility of simple chemical compounds acting as pheromones should not be overlooked or obscured by the use of multivariate statistics (Apps 2013).

Semiochemical perception

Odor detection in mammals is accomplished via two parallel-processing systems, the main olfactory epithelium and the vomeronasal organ located directly above the roof of the mouth (Dehnhard 2011). Olfactory stimuli reach the vomeronasal organ through the nasal cavity in rodents or through the nasopalatine canal connecting the oral and nasal cavities in carnivores, marsupials, and some primates (Dehnhard 2011). Traditionally, it was thought that the main olfactory system received input from airborne volatile compounds while the accessory olfactory system and the vomeronasal organ detected less volatile components and was responsible for the majority of pheromone perception (Dehnhard 2011). More recent studies have indicated that this proposed strict functional dichotomy is not as straightforward as previously thought. For some effects, the

important odorants are perceived by the main olfactory system while for others, the components responsible are perceived via the vomeronasal organ (Johns et al. 1978; Brennan and Keverne 2004; Gelez and Fabre-Nys 2006; Brennan and Kendrick 2006; Keller et al. 2009).

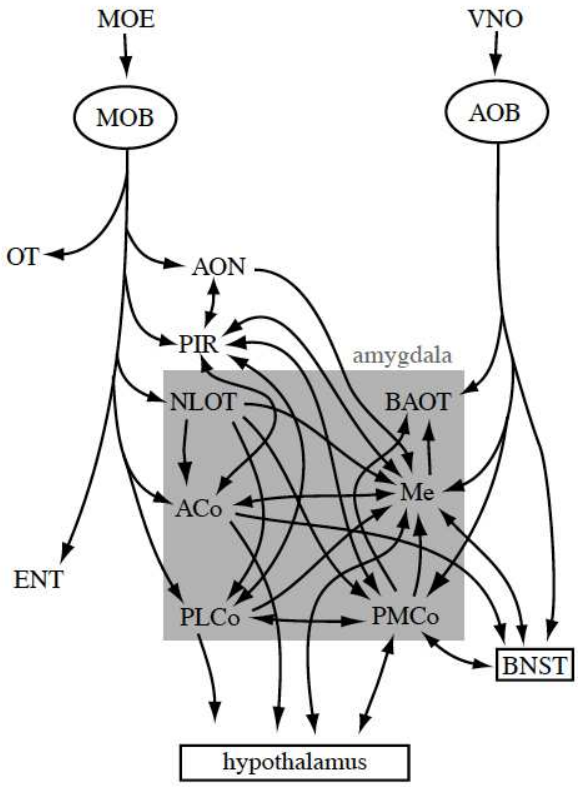


Figure 3. Schematic of the major projections of the main olfactory system and the vomeronasal system in the rat (*Rattus norvegicus*).
Abbreviations: ACo=anterior cortical nucleus; AOB=accessory olfactory bulb; AON=anterior olfactory nucleus; BAOT=bed nucleus of the accessory olfactory tract; BNST=bed nucleus of the stria terminalis; ENT=entorhinal cortex; Me=medial nucleus; MOB=main olfactory bulb; MOE=main olfactory epithelium; NLOT=nucleus of the lateral olfactory tract; OT=olfactory tubercle; PIR=piriform cortex; PMCo=posterior medial cortical nucleus; PLCo=posterior lateral cortical nucleus; VNO=vomeronasal organ (From Brennan and Kendrick 2006).

The vomeronasal organ's sensory neurons project into the accessory olfactory bulb while those of the main olfactory epithelium projection into the main olfactory bulb

(Figure 3). From there, the main olfactory system sends impulses into the amygdala, piriform cortex, orbitofrontal cortex, hippocampus, and hypothalamus, while the accessory olfactory bulb sends projections directly to the amygdala and then to the hypothalamus (Dulac and Torello 2003; Brennan and Keverne 2004; Brennan and Kendrick 2006; Kelliher and Wersinger 2008). Because of these widespread connections with other brain centers, the impulses from the vomeronasal organ and from the main olfactory system are well integrated with each other and also with other sensory inputs, allowing for great flexibility in emotional and behavioral responses (Brennan and Kendrick 2006).

Messages of mammalian secretions

The body of mammalian semiochemical research to date has shown that mammalian exocrine secretions vary depending on several factors: sex, individual identity, age, social status, reproductive status, and a wealth of other parameters (Wyatt 2005; Dehnhard 2011) (Table 2). Although many studies attempt to ascribe semiochemical status to any compounds identified in mammal excretions or secretions (Apps 2013), I will only use the term semiochemical to describe compounds known or suspected of containing a message.

Table 2. Selected semiochemical research in mammals. Semiochemicals related to reproduction are in bold type.

Species	Common Name	Odor source	Message	Citation
Ailuropoda melanoleuca	Giant panda	urine	**estrus status**	Swaisgood et al. 2002; Dehnhard et al
Ailuropoda melanoleuca	Giant panda	scent mark (anal gland, feces, urine, vaginal gland)	sex differences	Hagey and MacDonald 2003
Ailuropoda melanoleuca	Giant panda	urine	sex differences	Swaisgood et al. 1999
Ailuropoda melanoleuca	Giant panda	anogenital gland	sex differences, age differences, individual identity	Yuan et al. 2004; Zhang et al. 2008a
Bos taurus	Cow	urine	**1-iodoundecane and di-n-propylphthalate produced during estrus. Attractive to bulls**	Ramesh Kumar and Archunan 2001
Callithrix jacchus	Common marmoset	circumgenital gland	individual identity	Smith et al. 2001
Canis lupus	Gray wolf	urine	sex differences, seasonal differences, **sexual maturity, hormonal status**	Raymer et al. 1984; Raymer et al. 1986
Castor canadensis	Beaver	anal gland	relatedness	Sun and Müller-Schwarze 1998
Castor canadensis	Beaver	castoreum and anal gland secretion	individual identity, sex, and possibly social rank	Welsh and Müller-Schwarze 1989
Crocuta crocuta	Spotted hyena	anal gland	group membership	Burgener et al. 2008
Cryptoprocta ferox	Fossa	hair	sex differences	Vogler et al. 2008
Elephas maximus	Asian elephant	male temporal gland secretion	**Frontalin = musth state**, age differences	Rasmussen et al. 2002; Rasmussen and Greenwood 2003
Elephas maximus	Asian elephant	female urine	**(Z)-7-Dodecenyl acetate = pre-ovulatory pheromone. Males display flehmen and pre-copulatory behaviors in response**	Rasmussen 2001; Slade et al. 2003; Schulte et al. 2007
Equus ferus caballus	Horse	female urine	***m*- and *p*-cresol found in significantly higher concentrations during estrus and were proposed as indicators of ovulation****	Kimura 2001; Mozūraitis et al. 2012; Būda et al. 2012

**Būda et al. 2012 and Mozūraitis et al. 2012 directly contradict findings that p-cresol exists in higher concentration in non-estrous females and stallions than in estrous females (Kimura 2001)

Table 2 continued. Selected semiochemical research in mammals. Semiochemicals related to reproduction are in bold type.

Species	Common Name	Odor source	Message	Citation
Gulo gulo	Wolverine	anal gland	species differences	Wood et al. 2005
Gulo gulo	Wolverine	urine	individual identity	Wood et al. 2009
Heterocephalus glaber	Naked mole-rat	soiled nest material	group membership	O'Riain and Jarvis 1997
Lemur catta	Ring-tailed lemur	labial, scrotal, brachial gland	individual identity, seasonal differences, **fertility, pregnancy**	Scordato et al. 2007; Crawford et al. 2011; Kulahci et al. 2014; Crawford and Drea 2015
Loxodonta africana	African elephant		**reproductive status**	Bagley et al. 2006; Meyer et al. 2008
Loxodonta africana	African elephant	male urine	**musth state**, age differences	Rasmussen and Wittemyer 2002
Lutra lutra	Eurasian otter	anal gland	age differences, sex differences, **reproductive status**	Kean et al. 2011
Mandrillus sphinx	Mandrill	sternal gland	age differences, sex differences, rank, group membership	Setchell et al. 2010; Vaglio et al. 2015
Meles meles	Badger	subcaudal gland	individual identity is multivariate, group membership	Buesching et al. 2002
Microtus orchogaster	Prairie vole	direct naso-genital contact with male urine	**first estrus in virgin females**	Carter et al. 1980; Lyons and Getz 1993
Monodelphis domesticus	Gray short-tailed opossum	volatile and nonvolatile components of male suprasternal gland	**induces estrus and copulations** (Harder et al. 2008) increased body growth rate and **follicular development** (Harder and Jackson 2003)	Harder and Jackson 2003; Harder et al. 2008
Mus musculus	Mouse	male urine, preputial gland	individual identity, **sexual communication, advancing puberty onset, the maintenance of pregnancy, pregnancy blocking,** kin recognition**, timing of estrus**	Johns et al. 1978; Ma et al. 1999; Kayali-Sayadi et al. 2005; Morè 2006; Novotny et al. 2006; Kelliher and Wersinger 2008; Flanagan et al. 2011; Koyama et al. 2015
Mustela furo	Ferret	urine, anal gland	sex differences, individual identity, **reproductive season**	Zhang et al. 2005
Oryctolagus cuniculus	Rabbit	areola	2-methylbut-2-enal=nipple search pheromone	Schaal et al. 2003; Melo and González-Mariscal 2010

Table 2 continued. Selected semiochemical research in mammals. Semiochemicals related to reproduction are in bold type.

Species	Common Name	Odor source	Message	Citation
Panthera spp.	Asiatic lion, Sumatran tiger, Jaguar, Persian leopard	urine, marking fluid	cauxin-potenial enzyme to catalyze pheromone production	McLean et al. 2007
Phascolarctos cinereus	Koala	sternal gland	age differences, seasonal differences, **reproductive season**	Tobey et al. 2009
Sus scrofa	Boar	male saliva	**5α-androst-16-ene-3-one prompts reflexive lordosis in female**	Dehnhard 2011
Tachyglossus aculeatus	Short-beaked echidna	cloaca gland, spur	sex differences	Harris et al. 2012
Vulpes vulpes	Red fox	urine	induction of urine scent marking	Jorgensen et al. 1978; Whitten et al. 1980a)

Reproductive semiochemicals

Olfactory cues play an integral role in regulating a wide variety of reproductive functions from the onset of puberty, estrus synchronization, ovulation, kin identification, mate choice, and pregnancy maintenance or blocking (Brennan and Kendrick 2006; Melo and González-Mariscal 2010). Despite their obvious importance, these effects have been closely examined primarily in only a few traditional model species (Dehnhard 2011). Perhaps some of the most well-known effects have been investigated predominantly in the mouse (Table 2).

Despite the extraordinary efforts to characterize the compounds present in mammalian excretions and secretions, researchers have only been able to identify a total of 31 semiochemicals known to be responsible for certain responses in receiver individuals (Apps 2013). In the vast majority of semiochemical studies, researchers are forced to stop short of identifying compounds as semiochemicals due to the lack of bioassays to accompany the chemical identities of compounds found to be dependent on season, sex, or another biological parameter.

The male effect

The so-called "buck effect" or "male effect" where the presence of a male activates reproduction (estrus followed by spontaneous ovulation) in anestrous females via stimulation of the hypothalamic GnRH pathway has been primarily studied only in traditional farm animals (Bakker and Baum 2000; Dehnhard 2011; Murata et al. 2014). This effect is thought to have evolved in ungulates for estrus and ovulation synchronization to ensure offspring are born at the same time to maximize survival

(Dehnhard 2011). Interestingly, the components responsible for this effect seem to be shared between related species; exposure of female goats (*Capra aegagrus hircus*) to ram (*Ovis aries*) wool stimulated GnRH release suggesting that this signal is common to sheep and goats (Ichimaru et al. 2008). The male effect has been well described in reindeer (*Rangifer tarandus*); however, the role of pheromones was not discussed (Shipka et al. 2002). In a non-traditional species, the bush dog, exposure to a male decreases the inter-estrous interval of females (DeMatteo et al. 2006) suggesting that the male effect may be wide-spread through Mammalia.

Olfactory stimulation of ovulation

In mice and rats, ovulation can be induced by a male mounting without intromission and by exposing females to a novel cage previously occupied by a male (Johns et al. 1978), suggesting an olfactory channel of ovulation induction in these species. Females allowed contact with bedding sprayed with male urine ovulated at the same rate as females exposed to bedding from the male's enclosure (Johns et al. 1978). When major urinary proteins from male mice are applied to noses of estrous females, the number of eggs ovulated matches the number that are ovulated when females are exposed to whole male urine, suggesting that the major urinary proteins elicit the ovulation response in female mice (Morè 2006). This effect is not apparent in females lacking a vomeronasal organ (Morè 2006).

Olfactory effects of males on female ovulation are also seen in mink (*Mustela vison*) (Bakker and Baum 2000), goats (Bedos et al. 2010), and an olfactory cue prompting ovulation is hypothesized for the dromedary camel (*Camelus dromedarius*)

(Adams and Ratto 2013). The chemical compounds responsible for these effects and the mechanisms by which these substances operate remain uninvestigated.

Urinary compounds in Canidae

Within Canidae, the urinary constituents have been investigated for the red fox (Wilson et al. 1978; Jorgensen et al. 1978), coyote (Preti et al. 1976; Schultz et al. 1988), domestic dog (Goodwin et al. 1979; Kruse and Howard 1983; Schultz et al. 1985), gray wolf (Raymer et al. 1984; Raymer et al. 1986), African wild dog (Parker et al., 2010; Apps et al., 2012), and maned wolf (Childs-Sanford 2005; Goodwin et al. 2013).

For the gray wolf, the most common urinary compounds are ketones and reduced sulfur compounds (Raymer et al. 1984). In a study of red foxes, snow mounds were scented with a cocktail of eight synthetic urinary volatiles identified from conspecific urine (mostly ketones and sulfides) or with control compounds (Whitten et al. 1980a). Red foxes urine mark mounds with urinary volatiles significantly more frequently than control mounds, suggesting that ketones and reduced sulfur compounds are not only abundant but may also contain signals (Whitten et al. 1980a). Ketones are common in the urine of many mammals including canids (Raymer et al. 1984; Burger 2005). Reduced sulfur compounds are also found in high abundance in the urine of red foxes, coyotes, domestic dogs, gray wolves, and maned wolves (Wilson et al. 1978; Jorgensen et al. 1978; Raymer et al. 1984; Schultz et al. 1985; Schultz et al. 1988; Goodwin et al. 2013). Nitrogen-containing compounds such as amines are found in the anal gland secretions of red foxes, dogs, and coyotes (Albone and Perry 1975; Preti et al. 1976; Schultz et al. 1988), but are detected in low abundances in Iberian wolf (*Canis lupus signatus*) feces

(Martín et al. 2010). These compounds are entirely absent from gray wolf urine (Raymer et al. 1984) and African wild dog urine, feces, and glandular secretions (Apps et al. 2012).

About half of the compounds identified in African wild dog urine are also found in other canid secretions, although this species also shows a distinct difference from other canids in the almost complete lack of ketones, reduced sulfur compounds, and amines in its urine (Apps et al. 2012). Dimethyl sulfone, an oxidized sulfur compound, is a major component in African wild dog urine, but has not yet been found in other canid secretions (Apps et al. 2012).

Analyses of maned wolf urine show that sulfur-containing hemiterpenoids, hemiterpenoid alcohols, and nitrogen-containing pyrazines are main components contributing to this species' odiferous urine (Goodwin et al. 2013). Despite the fact that the urine of several other species of canid contains the reduced sulfur compound, 3-methyl-1-methylthiobut-3-ene, this compound is not found in the urine of the maned wolf. Instead, an isomer, 3-methyl-1-methylthiobut-2-ene is one of the most abundant urinary volatile in this species (Goodwin et al. 2013), further supporting evidence that urinary volatile compounds are unique to each species. The only other analysis of maned wolf urine (Childs-Sanford 2005) did not find any sulfur-containing substances, but was able to identify several of the pyrazine compounds common in maned wolf urine samples according to Goodwin et al. (2013).

For the gray wolf, urinary compounds vary with sex of the donor and with season (Raymer et al. 1984). Furthermore, the administration of testosterone to castrated male

gray wolves induces the production of urinary volatiles usually associated with intact males (Raymer et al. 1986), supporting the idea that urinary compounds are correlated with reproductive hormones and thus reflect reproductive status. This type of differential analysis ascribing putative semiochemical status to certain compounds has yet to be done for other species of canid, including for the maned wolf.

Research objectives

This dissertation is comprised of three major chapters connected by the overall theme of investigating chemical communication about reproduction in the maned wolf. The first study (Chapter 2) examined the typical composition of maned wolf urinary VOCs and assessed differences between male and female urinary VOCs. This chapter was published in Chemical Signals in Vertebrates XIII (Kester et al. 2014) and is reprinted here. The second study (Chapter 3) expanded on the findings of the first study through the analysis of a larger sample size and updated chemometric analyses. The third study (Chapter 4) assessed the hormonal and behavioral responses of female maned wolves to male urine.

Collectively this research represents a thorough investigation of urinary volatile compounds in the maned wolf and their roles as reproductive semiochemicals. Not only was this the most comprehensive characterization of the urinary volatile compounds for this species to date, but this dissertation also aimed to ascertain the biological function of several of the constituent compounds to determine the presence of ovulation induction via olfactory signals in the maned wolf. Induced ovulation is a relatively rare feature of Canidae and no carnivores studied to date have an olfactory mechanism underlying

ovulation. Therefore, this research addresses a critical knowledge gap in the reproductive biology of the maned wolf and the family Canidae. Further, the methods used here for putative pheromone discovery and bioassay testing have wide applicability to other species and conservation challenges. The methods of VOC extraction in Chapters 2 and 3 and the chemometric analyses in Chapter 3 provide a solid methodological foundation for future semiochemical studies in endangered species.

CHAPTER 2: AUTOMATED SOLID-PHASE MICROEXTRACTION OF URINARY VOCS FROM MANED WOLVES (*CHRYSOCYON BRACHYURUS*): A RECURSIVE WORKFLOW FOR GC-MS ANALYSIS

Abstract

The maned wolf (*Chrysocyon brachyurus*) is one of only two known canids exhibiting induced ovulation, a phenomenon that may be controlled by semiochemicals. This study employed headspace solid-phase microextraction with GC-MS coupled with the recursive use of Agilent's MassHunter Workstation and Mass Profiler Professional software to investigate the volatile organic compounds from monthly urine samples of eleven maned wolves. The goals were to find compounds that differ between males and females as a first step toward semiochemical discovery and to create a list of compounds found commonly across samples to form the basis of a control mixture for use in behavioral bioassays. Ten compounds were found to differ significantly between males and females ($P < 0.001$ and fold change >3.0): ψ-diosphenol, 1,3-di-tert-butylbenzene, and 2,4-dimethyl-1-heptene were respectively 3000, 600, and 85 times more abundant in males than females. Butanoic acid was unique to female maned wolves and nonanoic acid was 19 times more abundant in females than males. Twenty-five compounds were identified in >98% of the samples. Several of these compounds have been previously identified in maned wolf urine and some have been reported as semiochemicals in other mammal species. The analysis demonstrates that HS-SPME-GC-MS combined with automated data processing can successfully shorten the list of compounds that require

manual inspection and identification. The use of a recursive software workflow largely automates the search for maned wolf candidate semiochemicals, enabling an intense manual focus on compounds of interest.

Introduction

Maned wolf biology

The maned wolf (*Chrysocyon brachyurus*) is native to the tropical grassland habitats of South America (Dietz 1985). The species is currently listed as "Near Threatened" (Paula and DeMatteo 2015) with an estimated wild population of around 20,000 (Songsasen and Rodden 2010). Like most canids, maned wolves are monoestrous: females cycle only once per year (Asa and Valdespino 1998; Sillero-Zubiri et al. 2004). The onset of the breeding season appears to be a response to decreasing day length (Maia and Gouveia 2002; Rodden et al. 2007; Valdespino 2007), though several occurrences of breeding outside the typical season have been recorded (see below). For this species, breeding typically takes place from September through February in the northern hemisphere and from March through June in the southern hemisphere (Kleiman 1972; Rodden et al. 1996; Maia and Gouveia 2002; Rodden et al. 2007; Songsasen et al. 2014).

Maned wolves are induced ovulators; females enter estrus and/or ovulate only in the presence of a male (Songsasen et al. 2006; Johnson et al. 2014a). Although not all canid species have been investigated for this trait, as of yet the only other known induced ovulating canid is the Channel Island fox (*Urocyon littoralis*) (Asa et al. 2007). The domestic dog (*Canis familiaris*) (Concannon et al. 2009; Concannon 2011), gray wolf (*Canis lupus*) (Seal et al. 1979; Asa et al. 2006), coyote (*Canis latrans*) (Carlson and

Gese 2008), African wild dog (*Lycaon pictus*) (Monfort et al. 1997; Van der Weyde et al. 2015), bush dog (*Speothos venaticus*) (DeMatteo et al. 2006), red fox (*Vulpes vulpes*) (Mondain-Monval et al. 1977; Maurel et al. 1984), and arctic fox (*Alopex lagopus*) (Möller 1973) are known to be spontaneous ovulators.

Female maned wolves not paired with a male show baseline progestagen levels through the entire breeding season, demonstrating a lack of ovulation (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). Treating females with a gonadotropin releasing hormone (GnRH) agonist can successfully induce estrus and ovulation in paired females, but not in females housed singly (Johnson et al. 2014a). However, singleton females treated with the GnRH agonist followed by an exogenous luteinizing hormone treatment ovulate successfully (Johnson et al. 2014a).

Scent marking

Chemical signaling through scent marking is central to social recognition, territoriality, reproduction, and alarm signaling across Mammalia (Albone 1984; Burger 2005; Wyatt 2014). Scent marks continue to emit olfactory signals after the depositor has left, which makes scent-marking especially important for solitary animals that rarely come into direct contact with conspecifics (Hagey and MacDonald 2003; Delbarco-Trillo et al. 2011; Kean et al. 2011; Harris et al. 2012; Wyatt 2014).

Urine is considered to be a more important source of scent signaling than feces for canids (Gese and Ruff 1997; Parker 2010; Jordan et al. 2013). In general, canids urinate more frequently than they defecate, and they assume specialized postures for urination that are not necessary for urine elimination but are thought instead to provide a visual

signal to accompany the urine scent mark (Asa et al. 1984a; Pal 2003; Wirant et al. 2007). African wild dogs respond at higher rates to urine than to feces (Parker 2010; Jordan et al. 2013). Urine scent marking (but not defecation) increases in frequency during the breeding season in several canid species, including the maned wolf (Hradecky 1985; Asa et al. 1990; Rodden et al. 1996; Gese and Ruff 1997; Pal 2003; Parker 2010; Jordan et al. 2013). In maned wolves, the frequency of female urine scent marking is highest during proestrus and predicts reproductive success, as defined by the birth of pups (Rodden et al. 1996). Therefore, it is likely that maned wolves release signals in their urine leading up to breeding to communicate about reproduction eventually prompting the female to enter estrus and/or to ovulate.

Recent anecdotal evidence from individuals housed that the Smithsonian Conservation Biology Institute supports the role of a chemical signal. A female maned wolf ovulated when housed singly while sharing a fence line with a male. The female had visual access to the male as well as the ability to contact his urine scent marks (Johnson et al. 2014a). However, other females housed within sight of a male but with no contact with his urine failed to ovulate. These findings indicate that chemical signaling may be responsible for inducing estrus and/or ovulation in maned wolves.

Olfactory stimulation of estrus and/or ovulation in mammals

In laboratory mice (*Mus musculus*) estrus can be accelerated and ovulation can be induced by exposing group-housed females to male chemosignals (Marsden and Bronson 1964; Bronson and Whitten 1968; Whitten et al. 1968; Jemiolo et al. 1985; Jemiolo et al. 1986; Marchlewska-Koj et al. 1990; Ma et al. 1999; Morè 2006). When major urinary

proteins from male mice are applied to the noses of estrous females, the number of eggs ovulated is similar to the number that are ovulated when females are exposed to whole male urine, suggesting that major urinary proteins elicit the ovulation response in female mice (Morè 2006). A synchronizing effect of male urine is also seen for deermice (*Peromyscus maniculatus*) (Bronson and Marsden 1964) and rats (*Rattus norvegicus*) (Johns et al. 1978). Female rats allowed contact with male urine ovulate at the same rate as females exposed to bedding from the male's enclosure (Johns et al. 1978), demonstrating that male urine is the bioactive substance.

Olfactory effects of males on estrus and ovulation are documented in prairie voles (*Microtus ochrogaster*) (Carter et al. 1980), Siberian hamsters (*Phodopus sungorus*) (Dodge et al. 2002), sheep (*Ovis aries*) (Cohen-Tannoudji et al. 1989; Cohen-Tannoudji et al. 1994; Gelez et al. 2004), and goats (*Capra aegagrus hircus*) (Iwata et al. 2000; Murata et al. 2009; Bedos et al. 2010; Murata et al. 2014). Furthermore, an olfactory cue prompting ovulation is hypothesized for the dromedary camel (*Camelus dromedarius*) (Adams and Ratto 2013).

In carnivores, the so-called male effect is studied far less. There does seem to be a male effect in the bush dog, the closest living relative to the maned wolf, where the presence of an adult male decreases the inter-estrus interval of females (DeMatteo et al. 2006). However, to our knowledge, the compounds and mechanisms responsible for this effect in carnivores remain completely uninvestigated.

Maned wolf urinary volatile organic compounds

Because mammalian secretions are exceptionally complex (Burger 2005; Apps 2013), characterizing all the constituents that make up maned wolves' urine is a formidable task. Previously, only two studies of maned wolf urine have been published. They examined volatile organic compounds (VOCs) as likely candidates for signaling. In one study, Goodwin et al. (2013) found that sulfur-containing hemiterpenoids, hemiterpenoid alcohols, and pyrazines were the main components contributing to this species' odiferous urine. This study also supported the idea that different canid species have unique urinary VOCs; a compound found to be abundant in other canids, 3-methyl-1-methylthiobut-3-ene, was not found in maned wolves; whereas, an isomer tentatively identified as 3-methyl-1-methylthiobut-2-ene was abundant. The only other analysis (Childs-Sanford 2005) identified several of the pyrazine compounds also found by Goodwin et al. (2013).

Analysis of VOCs in urine samples typically employs gas chromatography-mass spectrometry (GC-MS) to identify compounds and measure their abundances. Even under the best conditions, chromatograms are crowded with peaks that can overlap or co-elute in such a way that some poorly-resolved compounds are hidden among those with higher abundance. Peaks for compounds with high abundance can be distorted, and peaks for compounds of low abundance may not be sufficiently resolved or distinguished from background noise. When peaks overlap, the mass spectrum at any given retention time (RT) may contain ions from one, two, or several compounds, thus making identification difficult and time consuming. Preliminary analysis (unpublished) of several maned wolf urine samples revealed around 800 peaks in each chromatogram after spectral

deconvolution. Even in a relatively small-scale experiment consisting of around 100 samples, the number of peaks quickly explodes to an intractable 80,000. With a rate of five min per peak working ten hours per day, checking all peak alignments manually would require 667 days.

In this study, a data-analysis software bundle was used to facilitate the analysis of GC-MS data from maned wolf urine samples. The strength of our data analysis method is that we will be able to conduct any sort of differential analysis to generate a list of candidate semiochemical compounds to be tested for biological relevance in behavioral bioassays. For these analyses we focused on searching specifically for two lists of compounds: those that differ between sexes and those that are common across maned wolf urine samples. Identifying compounds that differ between the sexes is a solid first step toward semiochemical candidate identification while generating a list of compounds present across the entire data set will be useful to create a synthetic maned wolf urine mixture to be used as a control or a vehicle for bioassay studies.

Methods

Animals

Eleven maned wolves housed at institutions within the United States were used in this study (Table 3). Monthly urine samples (4 – 15 mL) were collected when individuals urinated on a clean stainless steel pan placed in their enclosure. Each morning the pan was cleaned and the back of the pan (not the collecting side) was sprayed with around 0.5 mL of maned wolf urine, prompting individuals to urinate on the front of the pan. The samples were collected and frozen at -20°C within eight hours of elimination, a period of

time dictated by the logistics at the four participating institutions. This project was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent at each participating institution.

Table 3. Maned wolves that supplied urine samples.

Institution	SB#[a]	Sex	Age (years)
Connecticut's Beardsley Zoo, CT	3231	F	3
Connecticut's Beardsley Zoo, CT	3232	F	3
Fossil Rim Wildlife Center, TX	2845	F	7
Smithsonian Conservation Biology Institute, VA	2810	M	7
Smithsonian Conservation Biology Institute, VA	2844	M	7
Smithsonian Conservation Biology Institute, VA	2926	F	7
Smithsonian Conservation Biology Institute, VA	2954	M	7
Smithsonian Conservation Biology Institute, VA	3120	M	6
Smithsonian Conservation Biology Institute, VA	3184	F	3
White Oak Conservation Center, FL	2660	M	9
White Oak Conservation Center, FL	2945	F	7

[a]Studbook Number. Association of Zoos and Aquarium reference number of individual pedigree and demographic history.

Headspace solid-phase microextraction-gas chromatography-mass spectrometry

Urine samples were defrosted, vortexed, and 1.0 mL aliquots were dispensed into 10 mL glass headspace vials with metal twist caps and silicone/PTFE layered septa (La-Pha-Pack, Langerwehe, Germany). Prior to use vials were rinsed with ultrapure water, rinsed with methanol, and baked at 425°C overnight. Samples were saturated with high-purity sodium chloride to decrease the solubility of volatile organic compounds (Mills and Walker 2000). Samples were run in triplicate on a 7890A-5975C gas chromatograph-mass spectrometer (Agilent Technologies, Santa Clara, CA) fitted with a CombiPAL robotic sampling preparation and injection system (CTC Leap Technologies, Carrboro,

NC). The CombiPAL was configured with a solid-phase microextraction (SPME) adapter and a sample heater-agitator to automate the entire sample extraction and chromatography process. The instrument and autosampler system were controlled using MSD Chemstation software ver E.02.02 (Agilent Technologies, Santa Clara, CA).

Prior to running each sample batch, a 1 cm 50/30 μm divinyl benzene-carboxen-poly(dimethylsiloxane) (DVB/CAR/PDMS) stable-flex SPME fiber (Sigma-Aldrich, St. Louis, MO) was cleaned and preconditioned in a spare split-splitless inlet according to manufacturer recommendations of 270°C for 60 min. Following each run, the SPME fiber was cleaned for 20 min in this extra inlet prior to being re-used with another sample. VOCs in the sample were equilibrated between the headspace and urine at 500 rpm for 30 min at 37°C. Heating and agitation continued for 45 min while the SPME fiber was exposed to the headspace. The 50/30μm DVB/CAR/PDMS has been shown to extract the widest array of compound classes with the best sensitivity for commercial SPME fibers (Risticevic and Pawliszyn 2013).

Following extraction, compounds were thermally desorbed from the fiber in a temperature-programmable multi-mode GC inlet with a 0.75 mm ID SPME liner (Restek Corporation, Bellefonte, PA). The initial inlet temperature was 50°C for 0.12 min followed by a ramp to 240°C at 600°C min^{-1}. The column was a 0.25 mm ID by 30 m long RTX-VMS with a 1.0 μm film (Restek Corporation, Bellefonte, PA). The carrier gas was helium, and the initial GC oven temperature was 50°C for 3 min, followed by a ramp of 7°C min^{-1} to 240°C. Total run time was 45 min. The GC was equipped with Agilent's

backflush module, which reversed the column flow for 5 min following the 45 min GC run to eliminate sample-to-sample carry over and to maintain inertness of the flow path.

Retention-time locking was used to maintain consistent peak retention times, which facilitates identifications and comparisons of analytes across large sample sets.

The transfer line temperature was held at 290°C. The 5975C mass spectrometer operated with an electron energy of 70 eV in the full scan mode with a range from 40 to 350 m/z at a rate of 4.51 scans sec^{-1}. The ion source was at 300°C. The quadrupole was at 180°C.

Data analysis

Urine sample data were analyzed using MassHunter Workstation software (Agilent) including MassHunter Qualitative Analysis ver B.06.00 and MassHunter Quantitative Analysis ver B.07.00 for chromatographic data processing and Mass Profiler Professional ver 12.6.1 for visualization and statistical analysis. Subsequently these programs will be referred to as Qual, Quant, and MPP respectively. This software suite was originally developed for metabolomics and proteomics (Gu et al. 2011; Álvarez-Sánchez et al. 2012; Kim et al. 2014) but has applications in several fields: pharmaceutical impurity testing (West et al. 2010), food science (Bondia-Pons et al. 2014), as well as environmental toxicity studies (Hindle et al. 2013).

Qual analyzed all the samples as a batch with parameters selected in a Qual method. The program began the analysis by creating an ion chromatogram for every nominal ion. It integrated each ion chromatogram and created a peak list that was put through a deconvolution algorithm (a chromatographic covariance test to create

compounds from related ions that eluted at the same time) (Figure 4). Thus, for each compound there was an associated deconvoluted spectrum that contained far fewer ions from noise and adjacent peaks. The retention time window size factor was set to 80 and the extraction window was set to ± 0.3 AMU. Ions 73, 207, and 281 m/z were excluded since they represent GC column bleed. For each data file, the compounds were exported into a .CEF file for evaluation in MPP.

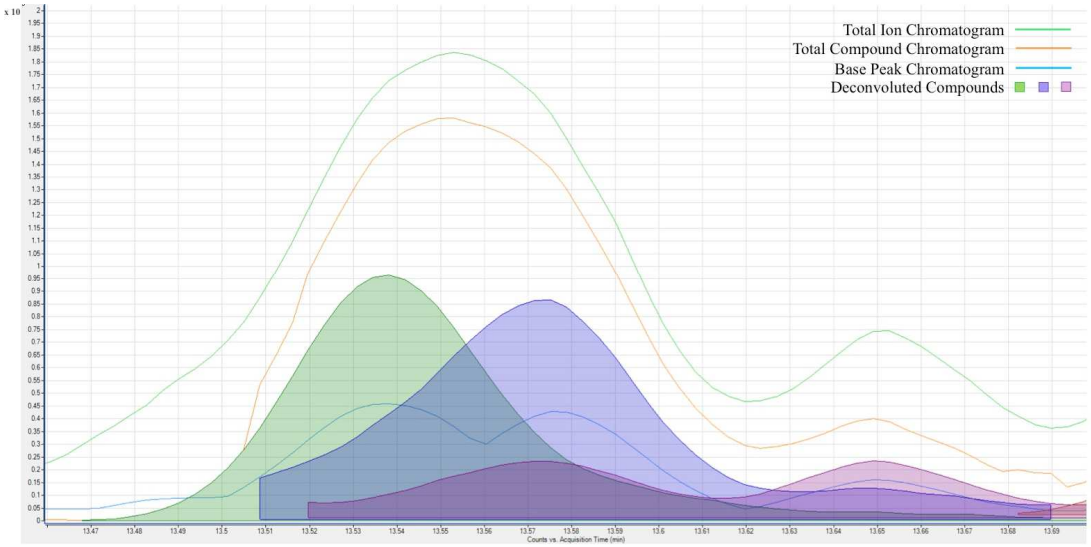


Figure 4. The deconvolution algorithm is able to separate overlapping, co-eluting compounds on the basis of individual ion chromatograms with high covariance. Total ion chromatogram shown in green, total compound chromatogram shown in orange, and base peak chromatogram shown in blue. Three overlapping, co-eluting compounds shown shaded.

MPP was then used to perform differential analyses to determine relationships among sample groups or experimental parameters. The software aligned each compound across the entire sample batch using an alignment algorithm that matched similar spectra at the same retention time. A retention time tolerance of ± 0.20 min was used along with

a spectral match factor of 0.30 and a delta m/z window of 0.20. The relative abundance values were then $\log_2$ transformed. Relative abundances of zero were set = 1E-5 so that the $\log_2$ value = -16.61 rather than being undefined (van den Berg et al. 2006).

Once compounds were aligned, the differential analyses could be performed across all samples with greater confidence. Compounds that potentially differed by sex were determined using a moderated *t*-test (Smyth 2004) on the $\log_2$ transformed relative abundances. Comparatively loose criteria were used in this step to minimize false negatives that could be present due to retention time shifts. Compounds with a corrected *P*-value < 0.05 and a fold change difference between the sexes of >2.0 were retained. Fold change between conditions A and B is calculated on the natural scale as condition A/condition B. Once on the $\log_2$ scale, $\log_2$(fold change) = $\log_2$(condition A) – $\log_2$(condition B).

To identify compounds that were found most commonly, the aligned peaks were filtered by frequency. Peaks initially found in >80% of data files were retained to minimize false negatives. The resulting two compound lists were far shorter than the total list of compounds across all samples and were exported as .CEF files for screening in Quant.

Each .CEF file from an MPP analysis formed a compound library for use in Quant to be used in a targeted analysis. The library contained the compounds identified by retention time, quantifier and qualifier ions, and ion ratios as a Quant method that was then applied to a batch containing all the raw sample data. The results were checked for proper base peak integration and particularly for correct compound assignments. False

negatives were corrected when some chromatographic peaks were assigned to one compound in some samples and another compound in other samples owing to retention time shifts (pers. obs.). False positives were eliminated when ion ratios clearly did not match the literature values or values in the NIST11 Mass Spectral Search Program ver. 2.0 (NIST11) (National Institute of Standards and Technology, Gaithersburg, MD).

At this point, compounds were identified by searching the NIST11 library. Further confirmation was conducted for those compounds where authentic standards were available. In the case of some pyrazines for which an authentic standard was not available, a small sample of peanut butter was run with the same HS-SPME-GC-MS method as pyrazines are common in roasted peanuts and peanut oil (Ku et al. 1998; Liu et al. 2011). The manually screened data were then exported into a .CEF file for final analysis.

Once again, MPP was used to perform visualizations and differential analyses using the $\log_2$ transformed relative abundances. Principal components analysis (PCA) allowed visualization of the similarities and differences between samples. PCA is a dimension reduction technique to visualize groupings of samples based on a combination of variables, in this case compounds (Wold et al. 1987). Each principal component is chosen to maximize the amount of variance it explains while being orthogonal to the other principal components. Finally, a moderated *t*-test and a fold change analysis offered insight into statistical significance for compounds that differed by sex. A compound was considered significantly different between groups if it met two criteria: a corrected *P*-

value of < 0.001 and a fold change between groups of >3.0. Numerical results are reported as mean ± standard deviation.

Results and discussion

A total of 103 urine samples from 11 wolves were analyzed, covering 6 – 12 months of 2013 with an average of 9.36 ± 2.06 months per individual. One sample with low volume (Female #3184, 9/11/2013) was run in duplicate rather than triplicate, resulting in a total of 308 data files.

Qualitative analysis, peak alignment, and preliminary analysis

Overall, 1682 compounds were aligned across the 308 data files. On average, samples showed 126.75 ± 24.92 compounds, meaning the vast majority of compounds were only found in one sample. The average relative standard deviation between replicates of each sample was 6.46%.

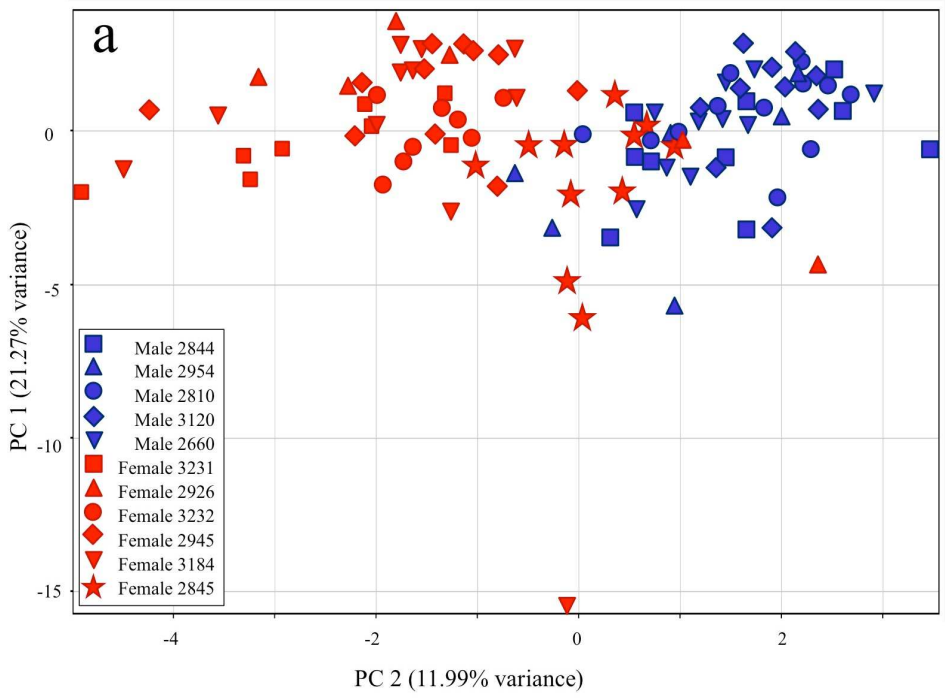
After compound alignment, the $\log_2$ of the relative abundance for each compound was reported for each of the samples. Based on these relative abundances, two lists were created: compounds that differed by sex with a $P < 0.05$ and a fold change between the sexes of >2.0 (63 compounds) and compounds occurring in >80% of samples (39 compounds). Each list described the compounds with chromatographic and mass-spectral parameters rather than with a compound name.

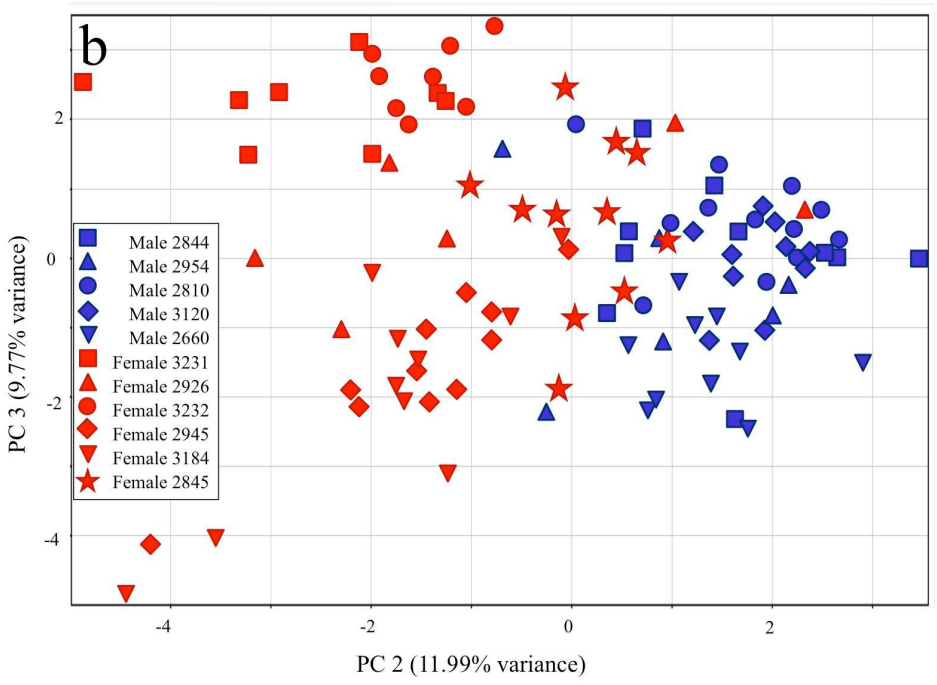
Compounds that differed by sex

For the analysis of compounds differing between sexes, false positives were removed and false negatives were added manually in Quant. The resulting candidate compound list contained 27 compounds.

The candidate list of 27 compounds generated from Quant was then imported back into MPP for a second round of statistical analysis since the preliminary statistical analysis occurred prior to manual checks of the integration and compound assignment verification (removal of false positives and addition of false negatives).

After averaging across replicates, PCA was used to visualize the grouping of the samples for the 27 compounds identified in Quant as differing by sex (Figure 5). In this analysis, three principal components explained 43.03% of variance between samples. Because principal component 1 (PC1) mainly described the variation attributable to the difference between the sample from Female 2845 on October 29, 2013 and the rest of the samples (Figure 5a), the PCA was re-run excluding this data point. Results remained stable with good separation between the sexes using three principal components. In both cases, principal component 2 (PC2) seemed to relate to sex with male wolves showing mainly positive scores and females showing negative scores (Figure 5a and Figure 5b). Principal component 3 (PC3) mainly described the variation between individuals (Figure 5b). The loadings plots (Figure 5c and Figure 5d) show the contribution of each compound to the three principal components.


a
PC 1 (21.27% variance)
PC 2 (11.99% variance)
0
-5
-10
-15
-4
-2
0
2
Male 2844
Male 2954
Male 2810
Male 3120
Male 2660
Female 3231
Female 2926
Female 3232
Female 2945
Female 3184
Female 2845


b
PC 3 (9.77% variance)
PC 2 (11.99% variance)
2
0
-2
-4
-4
-2
0
2
Male 2844
Male 2954
Male 2810
Male 3120
Male 2660
Female 3231
Female 2926
Female 3232
Female 2945
Female 3184
Female 2845

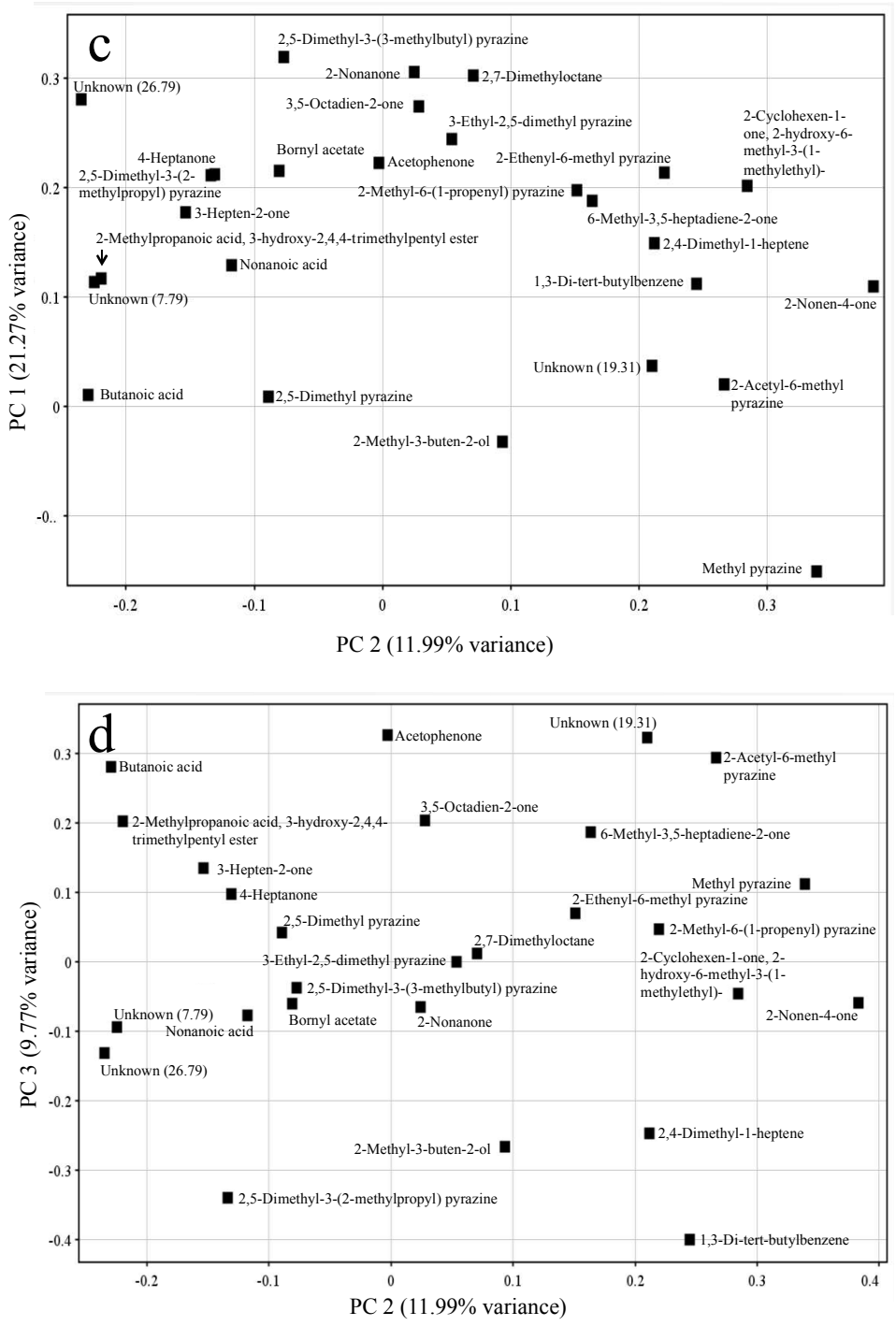


Figure 5. **(a) Principal component scores of 103 samples on principal components 1 and 2 using 27 compounds found to vary by sex. Samples are colored by sex and shaped by individual wolf. (b) Component loadings of 27 compounds on principal components 1 and 2. (c) Principal component scores of 103 samples on principal components 2 and 3 using 27 compounds found to vary by sex. (d) Component loadings of 27 compounds on principal components 2 and 3.**

Table 4. Compounds that differed in relative abundance between sexes in maned wolf urine.

Compound	RT	CAS No.	ID Method[a]	Proportion samples where present	
				Male (N = 48)	Female (N = 55)
2-Methyl-3-buten-2-ol[e]	4.20	115-18-4	NIST, STD	100.0%	100.0%
Unknown (7.79)	7.79			100.0%	100.0%
2,4-Dimethyl-1-heptene	8.22	19549-87-2	NIST	68.8%	50.9%
Methyl pyrazine[e]	9.50	109-08-0	NIST, STD	100.0%	100.0%
Butanoic acid[f]	10.35	107-92-6	NIST, STD	0.0%	16.4%
4-Heptanone	11.09	123-19-3	NIST, STD	100.0%	100.0%
2,5-Dimethyl pyrazine[e,f]	11.73	123-32-0	NIST, STD	100.0%	100.0%
2,7-Dimethyloctane	12.52	1072-16-8	NIST	100.0%	98.2%
2-Nonanone	12.61	821-55-6	NIST	100.0%	100.0%
3-Hepten-2-one	13.50	1119-44-4	NIST, STD	100.0%	100.0%
2-Ethenyl-6-methyl pyrazine[e]	14.47	13925-09-2	NIST, PB	100.0%	100.0%
3-Ethyl-2,5-dimethyl pyrazine[e]	15.52	13360-65-1	NIST, PB	100.0%	100.0%
2-Methyl-6-(1-propenyl) pyrazine	16.31	55138-67-5	NIST, PB	100.0%	100.0%
Acetophenone	16.93	98-86-2	NIST, STD	100.0%	100.0%
3,5-Octadien-2-one	17.67	38284-27-4	NIST	97.9%	98.2%
2-Nonen-4-one	17.85	32064-72-5	NIST	100.0%	100.0%
2-Acetyl-6-methyl pyrazine	17.92	22047-26-3	NIST, PB	100.0%	96.4%
6-Methyl-3,5-heptadiene-2-one	18.07	1604-28-0	NIST	100.0%	100.0%
2,5-Dimethyl-3-(2-methylpropyl) pyrazine	18.50	32736-94-0	NIST	100.0%	100.0%
1,3-Di-tert-butylbenzene	19.17	1014-60-4	NIST, STD	95.8%	69.1%
Unknown (19.31)	19.31			97.9%	94.5%
2-Cyclohexen-1-one, 2-hydroxy-6-methyl-3-(1-methylethyl)-	20.36	54783-36-7	NIST	97.9%	74.5%
2,5-Dimethyl-3-(3-methylbutyl) pyrazine	20.61	18433-98-2	NIST	100.0%	96.4%
(-)-Bornyl acetate	21.00	5655-61-8	NIST, STD	93.8%	85.5%
Nonanoic acid	21.41	112-05-0	NIST	14.6%	30.9%
2-Methylpropanoic acid, 3-hydroxy-2,4,4-trimethylpentyl ester	23.75	74367-34-3	NIST	100.0%	100.0%
Unknown (26.79)	26.79			100.0%	100.0%

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number

[a]Identification methods: NIST National Institute of Standards and Technology 2011 library of mass spectra; STD retention time match to authentic standard; PB retention time and mass spectral match to compound in peanut butter (see text).

Table 4 continued. Compounds that differed in relative abundance between sexes in maned wolf urine.

Compound	Log_2(relative abundance) mean ± SD		Fold Change		*P*	Significance[d]
	Male (N = 48)	Female (N = 55)	Raw[b]	Log_2[c]		
2-Methyl-3-buten-2-ol[e]	22.68 ± 1.13	22.08 ± 1.10	1.51	0.60	4.01E-05	
Unknown (7.79)	13.90 ± 0.87	14.48 ± 0.86	-1.5	-0.58	1.94E-07	
2,4-Dimethyl-1-heptene	1.45 ± 14.55	-4.96 ± 13.11	85.22	6.41	5.96E-04	***
Methyl pyrazine[e]	17.21 ± 1.24	16.24 ± 1.37	1.96	0.97	2.10E-09	
Butanoic acid[f]	-16.61 ± 0	-10.77 ± 13.27	-57.1	-5.84	2.14E-07	***
4-Heptanone	20.30 ± 2.13	22.04 ± 1.59	-3.35	-1.75	3.14E-12	***
2,5-Dimethyl pyrazine[e,f]	25.58 ± 1.33	25.96 ± 0.37	-1.3	-0.38	2.95E-03	
2,7-Dimethyloctane	14.26 ± 1.59	13.28 ± 4.24	1.97	0.98	1.07E-02	
2-Nonanone	15.63 ± 1.91	15.55 ± 3.87	1.06	0.08	8.41E-01	
3-Hepten-2-one	14.76 ± 1.66	16.12 ± 1.51	-2.57	-1.36	1.35E-10	
2-Ethenyl-6-methyl pyrazine[e]	21.93 ± 2.65	19.46 ± 2.3	5.53	2.47	2.38E-16	***
3-Ethyl-2,5-dimethyl pyrazine[e]	22.81 ± 2.14	22.85 ± 1.37	-1.02	-0.03	8.75E-01	
2-Methyl-6-(1-propenyl) pyrazine	22.64 ± 2.16	21.68 ± 2.21	1.95	0.96	1.76E-03	
Acetophenone	16.43 ± 2.63	17.06 ± 2.39	-1.55	-0.63	4.20E-02	
3,5-Octadien-2-one	12.01 ± 6.59	11.84 ± 5.59	1.13	0.17	8.23E-01	
2-Nonen-4-one	17.41 ± 2.61	11.96 ± 3.39	43.7	5.45	6.66E-28	***
2-Acetyl-6-methyl pyrazine	14.86 ± 1.86	10.80 ± 7.23	16.63	4.06	4.40E-09	***
6-Methyl-3,5-heptadiene-2-one	13.66 ± 2.42	10.94 ± 4.73	6.56	2.71	1.19E-05	***
2,5-Dimethyl-3-(2-methylpropyl) pyrazine	16.86 ± 1.63	17.25 ± 2.64	-1.31	-0.39	1.43E-01	
1,3-Di-tert-butylbenzene	15.60 ± 7.83	6.36 ± 15.8	603.86	9.24	7.31E-10	***
Unknown (19.31)	12.20 ± 4.65	10.91 ± 6.91	2.44	1.28	7.20E-02	
2-Cyclohexen-1-one, 2-hydroxy-6-methyl-3-(1-	11.70 ± 6.98	0.02 ± 11.74	3292.22	11.68	7.57E-17	***
2,5-Dimethyl-3-(3-methylbutyl) pyrazine	16.75 ± 1.84	16.10 ± 7.02	1.57	0.65	2.95E-01	
(-)-Bornyl acetate	9.99 ± 8.61	7.95 ± 11.33	4.11	2.04	9.26E-02	
Nonanoic acid	-14.20 ± 6.48	-9.94 ± 11.38	-19.21	-4.26	6.81E-04	***
2-Methylpropanoic acid, 3-hydroxy-2,4,4-	14.99 ± 2.03	15.94 ± 1.99	-1.93	-0.95	1.01E-04	
Unknown (26.79)	14.56 ± 1.16	15.92 ± 2.69	-2.57	-1.36	4.28E-06	

[b]Raw fold change = condition A/condition B

[c]In the log_2 scale, $log_2 (A / B) = log_2(A) – log_2(B)$

[d]Significance determined when $P < 0.001$ and |Fold change(raw relative abundance)| >3

[e]Also found in maned wolf urine by Goodwin et al. (2013)

[f]Also found in maned wolf urine by Childs-Sanford (2005)

There were seven compounds that showed significantly higher relative abundances in males (Table 4): ψ-diosphenol, (fold change (FC) = 3292.22), 1,3-di-tert-butylbenzene (FC = 603.86), 2,4-dimethyl-1-heptene (FC = 85.22), 2-nonen-4-one (FC = 43.70), 2-acetyl-6-methyl pyrazine (FC = 16.63), 6-methyl-3,5-heptadiene-2-one (FC = 6.56), and 2-ethenyl-6-methyl pyrazine (FC = 5.53). The compound ψ-diosphenol (synonym: 2-hydroxy-6-methyl-3-(1-methylethyl)-2-cyclohexen-1-one) is a terpenoid found in the essential oil of the buchu plant (*Agathosma betulina*), endemic to southern Africa (Fluck et al. 1961). 2,4-Dimethyl-1-heptene is a biomarker for bacterial infections, particularly for *Pseudomonas aeruginosa* (Sohrabi et al. 2014) and has also been identified as a byproduct of high-temperature pyrolysis of polypropylene (De Amorim et al. 1982). As far as we are aware, the above two compounds have not yet been reported in mammalian secretions.

Of the compounds shown to have significantly greater relative abundances in male maned wolves, two have been previously identified in mammalian secretions: 2-nonen-4-one is a putative reproductive semiochemical found in the rutting pits of male Alaskan moose (*Alces alces gigas*) (Whittle et al. 2000). Female moose roll in the pits that have been marked extensively with male urine. This compound is also a constituent in the urine of 67 out of 84 European badgers (*Meles meles*) (Service et al. 2001). 2-Ethenyl-6-methyl pyrazine has been previously identified in both mammals and insects. It is present in the urine of sexually intact male brown antechinus (*Antechinus stuartii*, family Dasyuridae) and was not identified in females (Toftegaards et al. 1999). 2-Ethenyl-6-methyl pyrazine is also a reproductive semiochemical in the papaya fruit fly

(*Toxotrypana curvicauda*) (Robledo and Arzuffi 2012). To our knowledge, this compound has not yet been identified in urine of carnivores.

Three compounds were reported in foods: 1,3-di-tert-butylbenzene is one of the main VOCs produced by the mycelium of *Tuber borchii*, an edible species of truffle (Tirillini et al. 2000). 2-Acetyl-6-methyl pyrazine is a common flavor compound responsible for the roasted aroma of grains, peanuts, and liquors (Buttery et al. 1997; Buttery et al. 1999; Fan et al. 2007; Liu et al. 2011). 6-Methyl-3,5-heptadiene-2-one is found in tomato products (Buttery et al. 1990), artichoke leaves (*Cynara scolymus L.*) (Saucier et al. 2014), and also is found in the essential oil of the herb *Hypericum annulatum* (Radulović et al. 2010) and species of *Erodium* (Stojanović-Radić et al. 2010), as well as paprika (*Capsicum annuum* L.) oleoresin (Guadayol et al. 1997).

One compound, butanoic acid, was found only in female maned wolves (Table 4), though not in all females tested. Butanoic acid seems to be common in secretions from other members of Canidae. This compound is present in anal gland secretions, feces, urine, and the preputial hair tufts of the African wild dog (Parker 2010; Apps et al. 2012), in the anal gland secretion and feces of black-backed jackal (*Canis mesomelas*) (Apps et al. 2012), and in relatively high abundance in the feces of the Iberian wolf (*Canis lupus signatus*) (Martín et al. 2010). This compound also is found in tiger (*Panthera tigris*) urine marks (Burger et al. 2008), in cloaca secretions of the Tasmanian short-beaked echidna (*Tachyglossus aculeatus setosus*) (Harris et al. 2012), in the circumanal gland of the marmoset monkey (*Callithrix jacchus*) (Smith et al. 2001), in the buccal gland of the

dwarf hamster (*Phodopus sungorus*) (Burger et al. 2001), and in milk of the European rabbit (*Oryctolagus cuniculus*) (Schaal et al. 2003).

Two compounds were significantly more abundant in female maned wolves than males (Table 4): nonanoic acid (FC = 19.21) and 4-heptanone (FC = 3.3). Nonanoic acid is present in a wide variety of mammalian secretions including the urine marks of tigers (Burger et al. 2008) and leopards (*Panthera pardus*) (Poddar-Sarkar and Brahmachary 2004), the urine of *Mus domesticus* and *Mus spicilegus* (Soini et al. 2009), anal gland secretion of the wolverine (*Gulo gulo*) (Wood et al. 2005) and black-backed jackal (Apps et al. 2012), the preputial hair tuft and urine of the African wild dog (Apps et al. 2012), and the cloaca secretion of the Tasmanian short-beaked echidna (Harris et al. 2012). As far as we are aware, differential analyses based on sex have not yet been done for any of the above species.

4-Heptanone has been previously identified in urine across a wide variety of mammals including the gray wolf (Raymer et al. 1986), wolverine (Wood et al. 2009), African wild dog (Parker 2010), and several species of strepsirrhine primate (Delbarco-Trillo et al. 2011). Differences in the abundance of 4-heptanone between the sexes are known for some species. In the cheetah (*Acinonyx jubatus*) this compound is only found in female urine (Burger et al. 2006), while in the ferret (*Mustela furo*) 4-heptanone is more abundant in males than in females (Zhang et al. 2005), and in the rat this compound is only found in male urine (Zhang et al. 2008b). In the red fox, 4-heptanone is identified in both males and females but there is a difference in abundance for males between breeding and non-breeding seasons (Jorgensen et al. 1978). Similarly, 4-heptanone

increases in concentration from non-breeding to breeding season in male white-tailed deer (*Odocoileus virginianus*) (Miller et al. 1998). This compound also occurs in the urine of African elephant (*Loxodonta africana*) females during both surges of the reproductive hormone, luteinizing hormone (Goodwin et al. 2005).

Compounds found commonly

Once false positives were removed and false negatives were added via the manual recursion process in Quant, the candidate list of compounds found commonly across samples was pared down from 39 to 25 compounds. The resulting compounds were found in >98% of the samples (Table 5). The compound found at a retention time of 11.4 min was likely 3-methyl-1-methylthiobut-2-ene for the reasons discussed in Goodwin et al. (2013). The ion relative abundance ratios in mass spectra here matched those described in that work. Most of the pyrazines and hemiterpenoids reported in Childs-Sanford (2005) and Goodwin et al. (2013) were also found here (Table 5).

To further analyze the 25 compounds found to be common across samples, heat maps were created to visualize the changes in compound abundance over the year for males and females separately. No discernible patterns were detected that would indicate changes in abundance of these compounds between breeding- and non-breeding seasons.

Table 5. Compounds common in maned wolf urine.

Compound	RT	CAS No.	ID Method[a]	Proportion Samples where present (N = 103)	Log_2(relative abundance) (mean ± SD) (N = 103)
2-Methyl-3-buten-2-ol[b]	4.25	115-18-4	NIST, STD	100.0%	22.19 ± 1.19
3-Methyl-2-buten-1-ol[b]	8.70	556-82-1	NIST, STD	100.0%	22.27 ± 1.07
3-Methyl-2-butenal	9.70	107-86-8	NIST, STD	100.0%	16.22 ± 1.26
4-Heptanone	11.20	123-19-3	NIST, STD	100.0%	21.03 ± 2.03
1-Methylthio-3-methylbut-2-ene[b]	11.40	NA		100.0%	19.07 ± 2.38
2,5-Dimethyl pyrazine[b,c]	11.80	123-32-0	NIST, STD	100.0%	25.89 ± 0.46
Trimethyl pyrazine[b,c]	13.82	14667-55-1	NIST, STD	100.0%	20.94 ± 1.05
3-Ethylcyclopentanone	14.17	10264-55-8	NIST	100.0%	15.71 ± 2.5
6-Methyl-5-hepten-2-one	14.35	110-93-0	NIST, STD	100.0%	16.03 ± 1.24
2-Ethenyl-6-methyl pyrazine[b]	14.49	13925-09-2	NIST, PB	100.0%	20.73 ± 2.62
2,5-Dimethyl-3-ethyl pyrazine[b]	15.57	13360-65-1	NIST, STD	100.0%	22.93 ± 1.36
2-Methyl-6-(1-propenyl) pyrazine[b,c]	16.40	18217-81-7	NIST	100.0%	22.5 ± 2.06
Acetophenone	17.04	98-86-2	NIST, STD	100.0%	17.9 ± 1.31
Unknown (17.40)	17.40			100.0%	14.92 ± 1.97
3,5-Dimethyl-2-propyl pyrazine	17.48	32350-16-6	NIST	100.0%	18.99 ± 1.85
2,6-Dimethylcyclohexanol	17.99	5337-72-4	NIST	100.0%	15.03 ± 1.55
2,5-Dimethyl-3-(2-methylpropyl) pyrazine	18.42	32736-94-0	NIST	100.0%	18.3 ± 2.29
2-Acetyl-3,5-dimethyl pyrazine	18.78	54300-08-2	NIST, STD	100.0%	17.34 ± 1.16
3-Butyl-2,5-dimethyl pyrazine	19.60	40790-29-2	NIST	98.0%	14.88 ± 5.38
β-Cyclocitral	20.20	432-25-7	NIST, STD	98.0%	13.75 ± 4.53
2,5-Dimethyl-3-(3-methylbutyl) pyrazine	21.00	18433-98-2	NIST	100.0%	19.39 ± 2.33
Unknown (21.79)	21.79			100.0%	18.73 ± 1.47
Unknown (21.96)	21.96			100.0%	16.64 ± 2.47
Geranyl nitrile	25.07	5146-66-7	NIST	100.0%	17.63 ± 3.5
3-Hydroxy-β -damascone	26.78	102488-09-5	NIST	100.0%	14.94 ± 2.28

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number

[a]Identification methods: NIST National Institute of Standards and Technology 2011 library of mass spectra; STD retention time match to authentic standard; PB retention time and mass spectral match to compound in peanut butter (see text).

[b]Also found in maned wolf urine by Goodwin et al. (2013)

[c]Also found in maned wolf urine by Childs-Sanford (2005).

Conclusions

The data analysis conducted with this multistep software method filtered a nearly unmanageable body of raw GC-MS sample data into a list of compounds that were shown to be of considerable interest to this study. The same data analysis protocols can be used with other differential analyses to provide a better understanding of various chemical signaling mechanisms and their associated semiochemicals in many other vertebrates.

The resulting list of putative semiochemicals includes both compounds that have been previously identified in maned wolves and in other species, and compounds that have not previously been identified in maned wolf urine or in other mammalian secretions. The compounds found to be common to all maned wolves in this study could prove important as a control or background signal in future bioassay research. It is possible that they confer non-reproductive signaling among conspecifics. Furthermore, the compounds that were shown to differ by sex are good candidates for semiochemical testing through bioassay research.

CHAPTER 3: PUTATIVE REPRODUCTIVE SEMIOCHEMICALS IN THE MANED WOLF (*CHRYSOCYON BRACHYURUS*)

Abstract

The maned wolf (*Chrysocyon brachyurus*) is an induced ovulator. Though the mechanism of ovulation induction remains unknown, it is suspected to be a urinary chemical signal. This study assessed volatile organic compounds (VOCs) in weekly urine samples across five months from thirteen maned wolves (six males, one castrated male, six females) using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). Eighteen VOCs differed significantly ($P < 0.001$ and fold change >3.0) between males and females. Using partial least squares discriminant analysis and random forests, the compounds with the highest influence on the sex classification were tentatively identified as 3-iodo-E-2-octenoic acid, 10-methyl-2-oxecanone, an unidentified compound, 1,1'-thiobis-cyclopentane, and 2-nonen-4-one. Fifty VOCs differed between intact males and a castrated male. Important classifier compounds were tentatively identified as tetrahydro-2-isopentyl-5-propyl furan, 2-methyl-6-(1-propenyl) pyrazine, and 2-nonen-4-one. All compounds that were differentially expressed by sex or male reproductive status (intact or castrated) were investigated for their temporal pattern in the individuals that successfully bred during the study. There were 15 VOCs in the breeding female that exhibited the same temporal pattern as each other (correlated) with a distinctive peak on the date of breeding and two VOCs where both the breeding male and

female showed a peak coincident with the date of copulation: 1,1'-thiobis-cyclopentane and 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one (tentative identities). Several VOCs established as important here have been implicated in reproductive communication in other mammals. This study provides the most comprehensive analysis of maned wolf urinary VOCs to date. Several putative semiochemicals were identified as good candidates for behavioral bioassays to determine their role in maned wolf reproduction and ascertain the identity of chemical signals involved in ovulation induction.

Introduction

The maned wolf (*Chrysocyon brachyurus*) is a unique member of Canidae (the dog family), endemic to tropical grasslands of South America (Dietz 1985). The species is listed by the IUCN RedList as "Near Threatened", with an estimated wild population of 17,000 (Paula and DeMatteo 2015). Intriguingly, the maned wolf seems to be an induced ovulator, meaning that females ovulate only in the presence of a male (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). As of yet the only other presumed induced ovulating canid is the Channel Island fox (*Urocyon littoralis*) (Asa et al. 2007). The domestic dog (*Canis familiaris*) (de Gier et al. 2006; Concannon et al. 2009; Concannon 2011), gray wolf (*Canis lupus*) (Seal et al. 1979; Asa et al. 2006), red wolf (*Canis rufus*) (Walker et al. 2002), coyote (*Canis latrans*) (Carlson and Gese 2008), African wild dog (*Lycaon pictus*) (Monfort et al. 1997; Van der Weyde et al. 2015), bush dog (*Speothos venaticus*) (DeMatteo et al. 2006), red fox (*Vulpes vulpes*) (Mondain-Monval et al. 1977; Maurel et al. 1984), and arctic fox (*Alopex lagopus*) (Möller 1973)

are spontaneous ovulators and other species of canid have yet to be investigated for this trait.

Female maned wolves housed without a male show baseline progestagen levels through the entire breeding season, indicating a lack of ovulation (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). A maned wolf pair that had been separated for the duration of the normal breeding season to prevent pregnancy copulated shortly after being reintroduced in April and gave birth more than two months outside the typical breeding and birthing season (Rodden et al. 2007). This suggests that the presence of a male strongly influences the timing of ovulation. In a recent observation, a female housed singly but sharing a fence line with a male ovulated (Johnson et al. 2014a). In this instance the female had visual access to the male as well as the ability to contact his urine scent marks deposited on the shared fence line. In the same reproductive season, several other females housed at the same facility, with visual contact to males failed to ovulate (Johnson et al. 2014a), suggesting visual stimulus is not sufficient and an olfactory mechanism involving urine underlies this phenomenon. In carnivores, evidence of olfactory signals prompting estrus and ovulation is far less prevalent than in other taxa (Morè 2006; Kelliher and Wersinger 2008; Ichimaru et al. 2008; Bedos et al. 2010). There does seem to be a male effect in the bush dog, the closest living relative to the maned wolf, where the presence of an adult male decreases the inter-estrus interval of females (DeMatteo et al. 2006). However, to our knowledge, the compounds and mechanisms responsible for this effect in carnivores remain completely uninvestigated.

Chemical communication is known to play an important role in mammalian behavior and reproductive processes for many species (Müller-Schwarze 2006; Dehnhard 2011). For the Canidae, urine is considered to be a more important source of scent signaling than feces (Gese and Ruff 1997; Parker 2010; Apps et al. 2012; Jordan et al. 2013). Urine scent marking, but not defecation, increases in frequency during the breeding season for several canid species including the maned wolf (Asa et al. 1984b; Hradecky 1985; Asa et al. 1990; Rodden et al. 1996; Gese and Ruff 1997; Pal 2003; Parker 2010; Jackson et al. 2012; Jordan et al. 2013). Maned wolf urine, like that of other canids, has a very distinctive pungent odor with scent marks remaining detectable to humans for several weeks (Emmons 2012; Goodwin et al. 2013). Rates of scent marking do not differ between male and female maned wolves (Songsasen et al. 2014). Within males, the frequency of urine scent marking is highest during proestrus compared to other stages of the reproductive cycle and is significantly higher in successful breeders when compared to unsuccessful individuals (Rodden et al. 1996). Similarly, females that bred successfully scent mark significantly more than those who are unsuccessful (Rodden et al. 1996).

Within Canidae, the urinary constituents have been characterized for the red fox (Wilson et al. 1978; Jorgensen et al. 1978; Whitten et al. 1980a), coyote (Preti et al. 1976; Schultz et al. 1988), domestic dog (Goodwin et al. 1979; Kruse and Howard 1983; Schultz et al. 1985), gray wolf (Raymer et al. 1984; Raymer et al. 1986), African wild dog (Parker 2010; Apps et al. 2012), and maned wolf (Childs-Sanford 2005; Goodwin et al. 2013). Analyses of maned wolf urine show that sulfur-containing hemiterpenoids,

hemiterpenoid alcohols, and nitrogen-containing pyrazines are main components contributing to this species' odiferous urine (Goodwin et al. 2013). Despite the fact that the urine of several other species of canid contains the reduced sulfur compound, 3-methyl-1-methylthiobut-3-ene, this compound was not found in the urine of the maned wolf. Instead, an isomer, 3-methyl-1-methylthiobut-2-ene is one of the most abundant urinary volatile compound (VOC) in this species (Goodwin et al. 2013), further supporting evidence that urinary VOCs are unique to each species. The only other analysis of maned wolf urine did not find any sulfur-containing compounds, but was able to identify several of the pyrazine compounds (Childs-Sanford 2005) common in maned wolf urine samples according to Goodwin et al. (2013). For the gray wolf, urinary compounds vary with sex of the donor and with season (Raymer et al. 1984). Furthermore, the administration of testosterone to castrated males gray wolves induces the production of urinary VOCs usually associated with intact males (Raymer et al. 1986), supporting the idea that urinary compounds are correlated with reproductive hormones, and thus, reflect reproductive status. This type of differential analysis ascribing putative semiochemical status to certain compounds has yet to be done for other species of canid, including for the maned wolf.

This study analyzed the differential expression of VOCs by sex, male reproductive status (intact or castrated), and by pairing status within both sexes. Then for successfully breeding individuals, temporal patterns were investigated for VOCs that were significantly different by sex or by male reproductive status. The hypotheses were that there are several VOCs that enable differentiation of sexes, male reproductive status

(intact or castrated), and pairing status and that some of these VOCs are temporally associated with breeding. The objective was to identify putative semiochemicals responsible for communication regarding reproduction. This work lays the foundation for categorizing the compounds responsible for ovulation induction in the maned wolf to better understand this unique reproductive mechanism within Canidae.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Smithsonian National Zoo and Conservation Biology Institute or its equivalent at each participating institution. Thirteen maned wolves (seven males, six females) (Table 6), were sampled for this study from August 1, 2014 to December 31, 2014. Animal care staff was unable to attribute samples from SB#3192 and SB#3195 to one particular wolf, so each urine sample was from one or potentially both brothers. As such, these brothers were treated as one wolf for the analyses. All other samples were from one, known maned wolf only and were not overmarked by other individuals housed within the same enclosure.

All wolves were fed a diet of custom maned wolf kibble (Mazuri, Land O'Lakes, Inc., Richmond, IN) supplemented with seasonal fruits and whole prey items (mice and guinea pigs). Water was available *ad libitum*. Urine samples (2 – 15 mL) were collected opportunistically, 1 – 2 times per week. Samples were collected within eight hours of elimination on a washed (water only) stainless steel urine catchment tray hung on the fence, typically for males, or laid on the ground, typically for females (Figure 6).

Table 6. Maned wolves that supplied urine samples.

SB#[a]	Age	Sex	Institution[b]	Housed with:	Breeding Information
3231	3	F	Beardsley	SB#3232	N/A Same sex sibling pair
3232	3	F	Beardsley	SB#3231	N/A Same sex sibling pair
2536	11	F	Buffalo	SB#3014	N/A Male neutered
3014	7	NeutM	Buffalo	SB#2536	N/A Male neutered
2845	8	F	Fossil Rim	***	None seen
3206	4	M	Fossil Rim	SB#3207 (sister)	N/A Female spayed
3192	3	M	Philadelphia	SB#3195	N/A Same sex sibling pair
3195	3	M	Philadelphia	SB#3192	N/A Same sex sibling pair
2810	8	M	SCBI	Single	N/A Single
2844	8	M	SCBI	SB#3184	None seen
3184	4	F	SCBI	SB#2844	None seen
2660	10	M	WOCC	SB#2945	Estimated breeding date = Oct 8, 2014
2945	8	F	WOCC	SB#2660	Estimated breeding date = Oct 8, 2014

[a]Studbook Number. Association of Zoos and Aquarium reference number of individual pedigree and demographic history.

[b]Beardsley: Beardsely Zoo, Bridgeport, CT; Buffalo: Buffalo Zoo, Buffalo, NY; Fossil Rim: Fossil Rim Wildlife Center, Glen Rose, TX; Philadelphia: Philadelphia Zoo, Philadelphia, PA; SCBI: Smithsonian Conservation Biology Institute, Front Royal, VA; WOCC: White Oak Conservation Center, Yulee, FL

***SB#2845 was housed with SB#3006 (male) and her 3 yearling pups from Aug. 1, 2014 – Sept. 9, 2014. SB#2845 was housed with her 3 yearling pups from Sept. 10 2014 – Dec. 31 2014

Alternatively samples were pipetted, using a disposable transfer pipet, from the concrete den floor immediately after elimination. To promote scent-marking behavior, occasionally <0.5 mL urine (human, domestic dog, or maned wolf) was sprayed on the reverse side of the catchment tray where an individual could detect it but where it would not contaminate the sample collection. Samples were collected in polypropylene centrifuge tubes (Corning, Inc., Tewksbury, MA) and were frozen at -20°C immediately after collection until processing.

Figure 6. Stainless steel urine catchment tray designed by Smithsonian Conservation Biology Institute to collect urine from large carnivores.

Sample preparation and GC-MS

Urine samples were defrosted but kept cold during the short time (~30 min) needed for processing. Samples were vortexed to homogenize then triplicates of 1 mL each were aliquoted into 10 mL glass headspace vials (BGB Analytik LLC, Alexandria, VA) with preslit PTFE-lined septa (BGB Analytik LLC, Alexandria, VA). Prior to use, vials were rinsed with ultrapure water, followed by methanol, and baked at 425°C overnight. If a sample was too small to create triplicates, duplicates were created (n = 16 samples run in duplicate). American Chemical Society (ACS)-grade sodium chloride (Thermo-Fisher Scientific, Hampton, NH) was added to each replicate to the point of saturation, evidenced by the fact that not all of it dissolved into the sample. Sodium

chloride was added to the sample to maintain stable ionic strength and to improve extraction efficiency of analytes (Pawliszyn 1999; Mills and Walker 2000). Finally 10 µL of a 15.3 µg/mL solution of naphthalene-d8 was added to each replicate as an internal standard to normalize chromatographic peak response.

VOCs were extracted from the headspace using solid-phase microextraction (SPME). Based on preliminary analyses, a 1 cm 50/30 µm divinyl benzene-carboxen-poly(dimethylsiloxane) (DVB/CAR/PDMS) stable-flex SPME fiber (Sigma-Aldrich, St. Louis, MO) was selected among eight SPME fiber coatings tested (Appendix 1: Optimization of headspace solid-phase microextraction for maned wolf urinary VOC analysis). This fiber coating extracts the widest array of compound classes with the best sensitivity for commercial SPME fibers (Risticevic and Pawliszyn 2013). Prior to analysis the SPME fiber was cleaned and conditioned in a spare GC split/splitless inlet at 270°C for 60 min. Following each run, the SPME fiber was cleaned for 30 min in this extra inlet prior to extracting the next sample.

Replicates were run on a 7890A-5975C gas chromatograph- mass spectrometer (Agilent Technologies, Santa Clara, CA) fitted with a Leap Technologies CombiPAL robotic sampling preparation and injection system (Autosampler Guys, Alexandria, VA). The CombiPAL was configured with a solid-phase microextraction (SPME) adapter, a cold-stack refrigerated sample compartment, and a sample heater-agitator to automate the entire sample extraction and injection process. The instrument and autosampler system were controlled using MSD Chemstation software ver E.02.02 (Agilent Technologies, Santa Clara, CA).

Samples were typically run in batches of ~60 replicates (20 samples). Vials were maintained at 4°C in the refrigerated sample compartment prior to extraction. Vials were then robotically transferred to the heater-agitator where they were stirred at 500 rpm at 37°C for 45 minutes in order to equilibrate VOCs with the vial headspace (gas above liquid urine). The SPME fiber was then exposed to the headspace while stirring and temperature were maintained in order to equilibrate VOCs with the DVB/CAR/PDMS sorbent layers of the SPME fiber. Following extraction, compounds were thermally desorbed in a temperature-programmable, multi-mode GC inlet containing a deactivated 0.75 mm ID SPME liner (Restek Corporation, Bellefonte, PA). The initial inlet temperature was 50°C for 0.12 min followed by a ramp to 270°C at 600°C min^{-1}.

The GC column was a 0.25 mm ID by 30 m long SUPELCOWAX 10 with a 0.25 μm film (Sigma-Aldrich, St. Louis, MO). The carrier gas was helium, and the initial GC oven temperature was 50°C for 3 min, followed by a ramp of 10°C min^{-1} to 100°C then 5°C min^{-1} up to 250°C. Column head pressure was determined by retention-time locking to assure consistent run-to-run retention times. Total run time was 38 min. The GC was equipped with Agilent's backflush module, which reversed the column flow for 5 min following the 38 min GC run to eliminate sample-to-sample carry over and to maintain inertness of the flow path.

The transfer line temperature was held at 290°C. The 5975C mass spectrometer operated with an electron energy of 70 eV in the full scan mode with a range from 40 to 350 m/z at a rate of 4.51 scans sec^{-1}. The ion source was at 300°C. The quadrupole was at 180°C.

Data analysis

Peak picking, grouping, and retention time correction in XCMS

The raw data files in MassHunter's .D format were converted into .mzXML format for data processing steps using ProteoWizard's MSConvert tool (Chambers et al. 2012). Files from replicates of each sample were processed together through the {xcms} R package (Tautenhahn et al. 2012) (see Appendix 2: Data analysis code for Chapter 3). This package contains functions to identify ion peaks, group the same ion peaks across replicates, correct retention time drift using the Obiwarp algorithm (Prince and Marcotte 2006), and then use the corrected retention times to regroup ion peaks across replicates. Next, missing ion peaks were filled in. Then a deconvolution algorithm in the {camera} R package (Kuhl et al. 2012) assigned ion peaks with the same peak shapes at the same retention times to the same compound to create a compound group made up of component ions.

Peak area averaging and quantitative ion selection

Using a custom Python script, ion peak areas were averaged across replicates of each sample, creating one mean value per urine sample for each ion. Then within each compound group, only the peak area of the ion with the largest average area, called the quantitative ion, was retained. This value is representative of the abundance of the VOC in that sample.

Normalization and scaling

Resulting processed data were analyzed using the web-based platform MetaboAnalyst (Xia and Wishart 2011). Quantitative ion peaks were aligned across samples (m/z tolerance = 0.25, RT tolerance = 1 s). Ion peaks found in fewer than half of

the samples per group (e.g. male or female) were excluded from further analysis because the goal of these differential analyses was to identify group differences. Missing data (i.e., VOCs that were not found in a given sample) were replaced with a low value for peak area equal to half the minimum observed value in the overall dataset. To minimize the number of non-informative VOCs analyzed and thereby increase the power of remaining analyses, an interquartile range filter was applied (Hackstadt and Hess 2009). This step excluded VOCs with low likelihood of being differentially expressed from downstream analysis, namely compounds with low abundances across the dataset and those with the same intensity across the dataset. VOC abundances were then normalized to the median and to the abundance of the internal standard. VOC abundances were generalized log transformed (Durbin et al. 2002) and pareto scaled (van den Berg et al. 2006).

Statistical Analysis and classification

Partial least squares-discriminant analysis (PLS-DA) and random forests (RF) were used to determine which VOCs contributed to the classification of samples as originating from different groups or classes. Comparisons were male versus female, intact versus castrated male, and paired versus unpaired individuals. PLS-DA is a supervised approach that uses the abundance of each VOC to maximize the separation between the different classes in the first few dimensions (latent variables) (Barker and Rayens 2003; Xia and Wishart 2011). These latent variables are ranked by how well they explain the variance of the classes. To validate the models, a Q^2 parameter was calculated as a measure for class prediction ability using 10-fold cross validation (Westerhuis et al.

2008; Worley and Powers 2012). The Q^2 value was then compared to the distribution of Q^2 values obtained from models of the same data using random permutations of class labels. This way statistical significance (*P*-values) of the given classification model can be obtained (Westerhuis et al. 2008; Worley and Powers 2012).

Random Forests (RF) is a classification technique that creates an ensemble of classification trees (500 in this case) in order to separate classes (Breiman et al. 1984; Breiman 2001; Liaw and Wiener 2002). Class prediction is based on the majority vote of the ensemble. Each tree was grown by first selecting a random subset of data; in this case 66% of the data was used to grow each tree. At each node, a randomly selected subset of seven VOCs was selected and the best classifier among those was chosen as a split (Breiman et al. 1984; Breiman 2001; Liaw and Wiener 2002). The process continued for each tree until all VOCs had been incorporated. The held-out data (33%) were then used as a test sample to obtain an unbiased estimate of the classification error, called the out-of-bag error or OOB error (Breiman et al. 1984; Breiman 2001; Liaw and Wiener 2002).

To determine which VOCs were most responsible for classification model, a variable importance in projection score (VIP) for PLS-DA and a mean decrease in accuracy score for RF were calculated for each VOC (Wold et al. 2001; Chong and Jun 2005). VOCs with higher VIP or higher mean decrease in accuracy contribute more to the classification model. In PLS-DA, the VIP is calculated as a weighted sum of the squared correlations between the PLS-DA latent variables and the original variable (Wold et al. 2001; Chong and Jun 2005). The weights correspond to the percentage of variation explained by the PLS-DA latent variable in the model. In RF, the mean decrease in

accuracy is calculated using the increase of the OOB error when that VOC is excluded from the classification model.

The differentially expressed VOCs were identified using the National Institute of Standards and Technology (NIST, Gaithersburg, MD) Automated Mass Spectral Deconvolution and Identification System (AMDIS, ver. 2.69) software and mass spectral library (NIST11). Deconvolution is a process that measures slopes and retention times of ion peaks in a chromatogram. Ions peaks that align are grouped together into deconvoluted spectra that exclude ions that coelute but do not match peak shapes and retention times. These "cleaned" spectra are library-searched for compound identification. Only compounds with 70% or greater probability of match to a molecule in the spectral library were named, otherwise compounds were labeled as "unknown" with a given quantitative ion and retention time.

Results

Overall, 332 urine samples and a total of 941 replicates were analyzed using headspace SPME and GC-MS. This corresponds to an average of 26.14 ± 7.11 (SD) samples per wolf.

Differences between male and female urinary VOCs

Following peak picking and grouping procedures, 74 peaks, each representing a VOC, were analyzed for differential expression between intact males (n = 6 individuals, 141 samples) and females (n = 5 individuals, 161 samples). Eighteen urinary VOCs were found to differ significantly ($P < 0.001$ and fold change >3.0) between males and females (Table 7). Of those, eleven VOCs were higher in abundance in males than females and

the other seven showed higher abundance in females than males. In the PLS-DA, the 74 VOCs were reduced to five PLS latent variables with good discrimination ability between males and females ($Q^2 = 0.92$, permuted $P < 0.001$) (Figure 7). The first latent variable, accounting for 14.9% of the explained variance, related to the difference in males compared to females. Compounds that were higher in abundance in female urine had negative loadings, while compounds that were higher in male urine had positive loadings. In the RF model, 159/161 samples were correctly classified as female and 139/141 samples were correctly classified as male, resulting in an OOB error = 0.0132. Variables considered most influential to the classification were essentially the same between the PLS-DA and the RF algorithms. The top five compounds were the same across both algorithms; these were: 3-iodo-E-2-octenoic acid, 10-methyl-2-oxecanone, the unknown 98 at 29.25 min, 1,1'-thiobis-cyclopentane, and 2-nonen-4-one (Figure 8).

Table 7. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between male (n = 141 samples) and female (n = 161 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Male (N = 141)	Female (N = 161)	Raw[b]	Log_2[c]			
3-iodo-E-2-octenoic acid	26.46	NIST ID: 308875	1.94 ± 0.83	-1.70 ± 0.79	54.99	-5.78	8.25E-120	3.29	0.154
unknown 98@29.25	29.25		1.37 ± 1.39	-1.20 ± 0.62	39.64	-5.31	2.53E-61	2.31	0.062
10-methyl-2-oxecanone	28.61	65371-24-6	1.66 ± 1.27	-1.45 ± 1.15	35.08	-5.13	5.77E-66	2.81	0.077
2-nonen-4-one	12.51	32064-72-5	1.19 ± 1.30	-1.04 ± 1.35	9.3	-3.22	6.20E-37	2.02	0.038
methyl 1-methyl-2-butenyl sulfide	19.18	89534-73-6	0.58 ± 1.87	-0.51 ± 1.69	8.07	-3.01	2.70E-07	0.98	0.002
1,1'-thiobis-cyclopentane	14.47	1126-65-4	1.23 ± 1.32	-1.08 ± 1.49	8.01	-3.00	5.33E-35	2.08	0.020
3-methyl-3-nitrobut-1-ene	21.35	1809-67-2	1.16 ± 1.79	-1.01 ± 1.64	5.82	-2.54	7.88E-24	1.96	0.015
2-methyl-3-buten-2-ol	3.86	115-18-4	1.18 ± 1.58	-1.03 ± 1.30	5.3	-2.41	3.71E-32	2.00	0.007
3-methyl-2-butene-1-thiol	27.36	5287-45-6	0.79 ± 1.94	-0.69 ± 1.76	5.25	-2.39	2.78E-11	1.34	0.002
3-hepten-2-one	8.91	1119-44-4	-0.78 ± 1.06	0.69 ± 1.66	5.19	2.38	1.94E-17	1.33	0.005
unknown 67@13.68	13.68		-0.87 ± 1.63	0.76 ± 1.53	5.06	2.34	5.09E-17	1.47	0.004
2,4-bis(1,1-dimethylethyl)-phenol	29.55	96-76-4	-0.46 ± 1.18	0.40 ± 1.60	5.01	2.32	2.05E-07	0.77	0.001
2-ethenyl-6-methyl-pyrazine	12.47	13925-09-2	0.80 ± 1.55	-0.70 ± 1.56	4.74	-2.25	3.09E-15	1.35	0.005
3-ethyl-2,5-dimethyl-pyrazine	11.89	13360-65-1	-1.04 ± 1.56	0.91 ± 1.76	3.68	1.88	4.19E-21	1.77	0.008
acetophenone	15.97	98-86-2	-0.40 ± 0.96	0.35± 1.56	3.57	1.84	9.39E-07	0.68	0.006
3-butyl-2,5-dimethyl-pyrazine	15.01	40790-29-2	1.08 ± 1.56	-0.94 ± 1.57	3.55	-1.83	1.81E-24	1.82	0.006
(1-hydroxycyclohexyl)phenyl-methanone	35.21	947-19-3	-0.44 ± 1.25	0.39 ± 1.47	3.48	1.80	2.38E-07	0.75	0.001
4-heptanone	5.88	123-19-3	-0.78 ± 1.93	0.68 ± 1.31	3.26	1.70	1.38E-13	1.32	0.012

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number; VIP = Variable Importance in Projection; PLS = Partial Least Squares; RF = Random Forests

[a]All compounds were identified by spectral library search with match probability >70%

[b]Raw Fold change = abundance(condition A)/abundance(condition B)

[c]Log_2 Fold Change = log_2(abundance(condition A) – log_2(abundance(condition B))

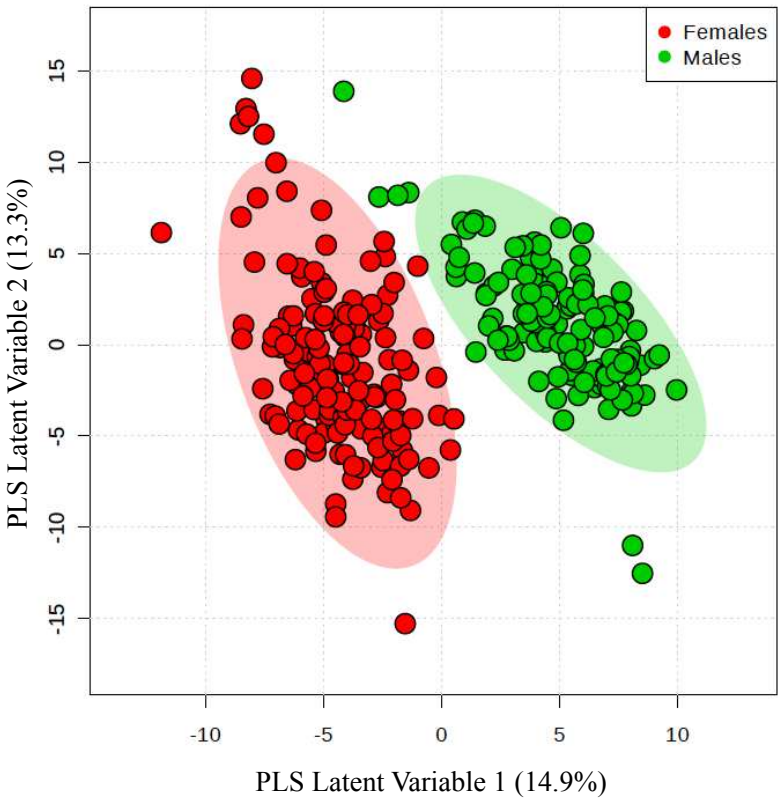


Figure 7. Scores on first 2 latent variables from partial least squares-discriminant analysis shown for female samples (n = 161) in red and male samples (n = 141) in green. Variance explained by each latent variable is shown in brackets.

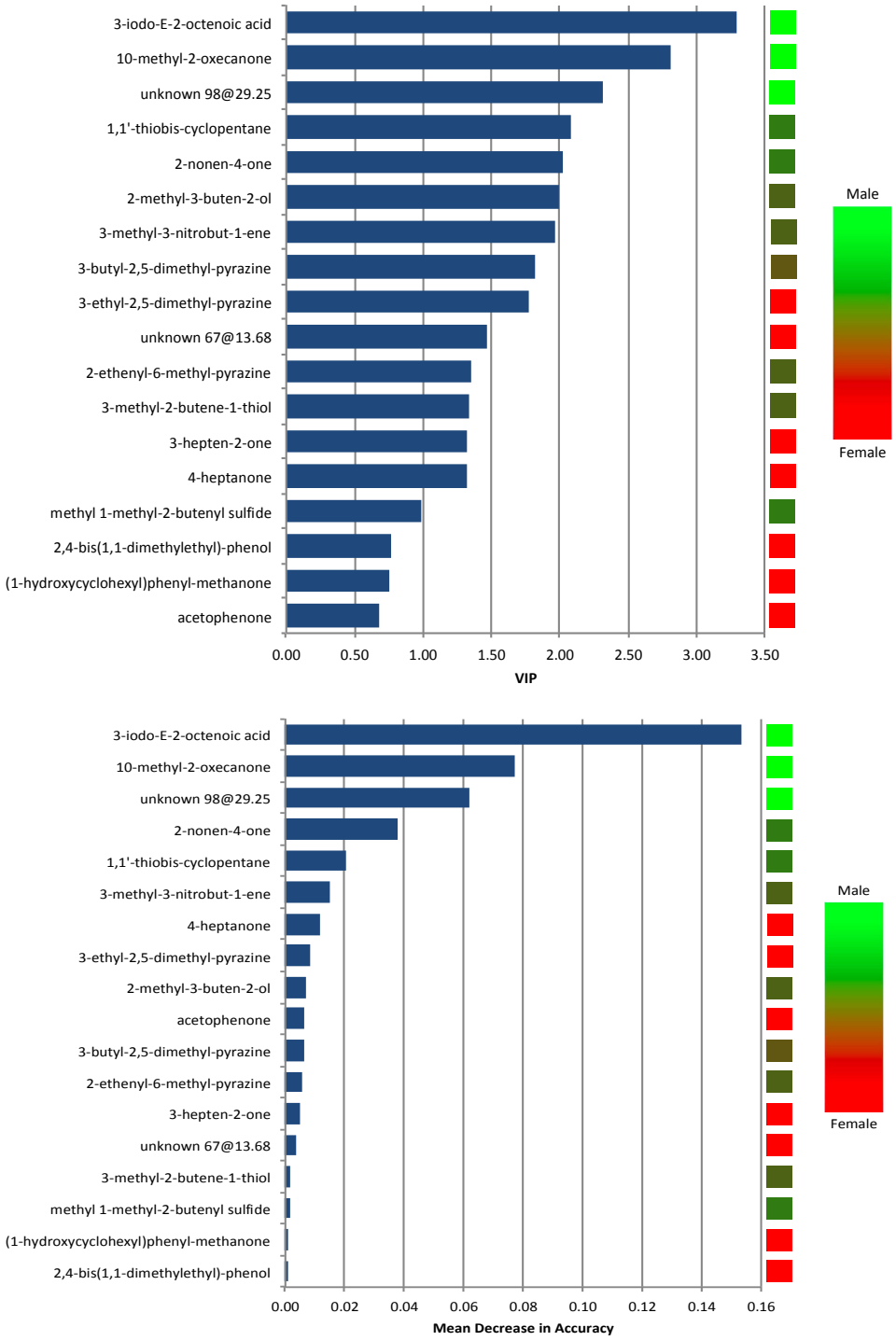


Figure 8. (a) Variable influence on projection (VIP) scores on partial least squares latent variable 1 and (b) mean decrease in classification accuracy for random forests model when each VOC is excluded for 18 influential VOCs in a comparison of males (n = 141 samples) and females (n = 161 samples). Boxes on right indicate the relative abundance of each VOC from more abundant in males (green) to more abundant in females (red).

Differences between intact and castrated male urinary VOCs

Following peak picking and grouping procedures, 101 peaks each representing a VOC were analyzed for differential expression between intact males (n = 6 individuals, 141 samples) and a castrated male (n = 1 individual, 15 samples). Out of those 101 peaks, 50 urinary VOCs were found to differ significantly ($P < 0.001$ and fold change >3.0) between intact and castrated males (Table 8). Of those, 24 VOCs were higher in abundance in intact males than castrated and the other 26 showed higher abundance in the castrated male samples than in those from intact males. In the PLS-DA, the 101 VOCs were reduced to three PLS latent variables with good discrimination ability between intact and castrated males ($Q^2 = 0.93$, permuted $P < 0.001$) (Figure 9). The first latent variable, accounting for 22.3% of the explained variance, related to the difference in intact males compared to castrated males. Compounds that were higher in abundance in urine from castrated males had negative loadings, while compounds that were higher in abundance in intact males' urine had positive loadings. In the RF model, 14/15 samples were correctly classified as castrated male and 140/141 samples were correctly classified as intact male, resulting in an OOB error = 0.0128. Variables considered most influential to the classification were very similar between the PLS-DA and the RF algorithms; nine of the top ten compounds were the same for each method. Compounds that appeared in the top five classifiers in both algorithms were: tetrahydro-2-isopentyl-5-propyl furan, 2-methyl-6-(1-propenyl) pyrazine, and 2-nonen-4-one (Figure 10).

Table 8. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between intact male (n = 141 samples) and castrated male (n = 15 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Intact Male (N = 141)	Castrated Male (N = 15)	Raw[b]	Log_2[c]			
2,5-dimethyl pyrazine	9.31	123-32-0	0.27 ± 2.65	-2.58 ± 0.57	4844.06	-12.24	3.09E-18	1.259	2.05E-04
3-methyl-3-nitrobut-1-ene	21.36	1809-67-2	0.36 ± 1.78	-3.40 ± 0.74	2944.89	-11.52	2.65E-17	1.662	1.21E-03
2-nonen-4-one	12.48	32064-72-5	0.41 ± 1.27	-3.85 ± 0.94	1125.23	-10.14	7.57E-13	1.879	1.29E-02
1,1'-thiobis-cyclopentane	14.48	1126-65-4	0.37 ± 1.38	-3.47 ± 0.86	234.61	-7.87	2.13E-13	1.693	2.91E-03
2,5-dimethyl-3-propyl pyrazine	12.97	18433-97-1	0.4 ± 1.19	-3.77 ± 0.99	142.23	-7.15	6.64E-12	1.841	3.01E-03
3-methyl-2-butene-1-thiol	27.37	5287-45-6	0.24 ± 1.94	-2.21 ± 0.8	138.59	-7.11	4.36E-11	1.081	4.23E-05
2-methyl-6-(1-propenyl) pyrazine	13.57	18217-81-7	0.45 ± 0.77	-4.20 ± 1.05	124.33	-6.96	2.14E-11	2.050	1.42E-02
tetrahydro-2-isopentyl-5-propyl furan	23.16	33933-71-0	0.43 ± 0.91	-4.04 ± 0.94	124.24	-6.96	2.87E-12	1.972	1.48E-02
3-ethyl 2,5-dimethyl pyrazine	11.57	13360-65-1	0.46 ± 0.94	-4.31 ± 1.80	114.73	-6.84	5.05E-08	2.103	8.66E-03
methyl 1-methyl-2-butenyl sulfide	19.18	89534-73-6	0.20 ± 1.85	-1.90 ± 0.80	104.66	-6.71	2.01E-09	0.930	1.08E-04
3-iodo-E-2-octenoic acid	26.47	NIST ID: 308875	0.40 ± 1.03	-3.80 ± 1.01	101.97	-6.67	1.67E-11	1.858	1.47E-02
1-(2-hydroxy-5-methylphenyl)-2-buten-1-one	19.09	5631-63-0	0.41 ± 0.95	-3.85 ± 0.96	77.09	-6.27	6.89E-12	1.879	9.66E-03
3,5-dimethyl-2-propyl pyrazine	10.99	32350-16-6	0.20 ± 2.00	-1.91 ± 0.75	70.47	-6.14	3.54E-10	0.932	3.52E-05
2,5-dimethyl-3-(3-methylbutyl)-pyrazine	16.05	18433-98-2	0.29 ± 1.62	-2.69 ± 0.84	58.69	-5.88	6.38E-12	1.316	3.93E-04
±-β,β-dimethyl-γ-(hydroxy-methyl)-γ-butyrolactone	19.36	52398-48-8	0.39 ± 0.83	-3.62 ± 1.04	45.14	-5.50	1.31E-10	1.769	1.16E-02
3-butyl-2,5-dimethyl-pyrazine	15.01	40790-29-2	0.26 ± 1.65	-2.47 ± 0.85	43.26	-5.43	4.48E-11	1.205	9.69E-05
10-methyl-2-oxecanone	28.61	65371-24-6	0.35 ± 1.42	-3.27 ± 1.26	40.3	-5.33	4.52E-09	1.597	1.98E-03

Table 8 continued. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between intact male (n = 141 samples) and castrated male (n = 15 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Intact Male (N = 141)	Castrated Male (N = 15)	Raw[b]	Log_2[c]			
unknown 175@26.32	26.32		0.22 ± 1.63	-2.08 ± 1.09	37.51	-5.23	2.70E-07	1.018	6.26E-05
1-methoxy-3-methyl-2-butene	3.25	22093-99-8	0.15 ± 1.96	-1.45 ± 0.78	32.81	-5.04	3.49E-07	0.709	3.65E-04
2-methyl-3-buten-2-ol	3.86	115-18-4	0.23 ± 1.66	-2.14 ± 0.87	27.29	-4.77	1.57E-09	1.045	3.06E-04
4-methyl-2-heptanone	7.36	6137-06-0	-0.29 ± 0.94	2.69 ± 2.32	26.23	4.71	2.02E-04	1.315	5.66E-04
2-acetyl-3,5-dimethylpyrazine	16.44	54300-08-2	0.32 ± 1.09	-3.04 ± 1.06	24.96	-4.64	1.35E-09	1.486	4.21E-03
tetrahydro-2,5-dimethyl-2H-pyranmethanol	10.93	54004-46-5	-0.25 ± 1.09	2.32 ± 1.73	16.79	4.07	4.36E-05	1.135	1.39E-04
3-methyl-2-buten-1-ol	9.25	556-82-1	0.41 ± 0.71	-3.87 ± 2.21	14.45	-3.85	2.66E-06	1.890	1.10E-02
3-hepten-2-one	8.93	1119-44-4	-0.22 ± 1.15	2.05 ± 2.06	13.55	3.76	7.88E-04	1.002	1.48E-04
N,N-dibutyl-formamide	18.58	761-65-9	-0.24 ± 1.03	2.25 ± 1.68	13.01	3.70	4.78E-05	1.097	8.26E-04
nonanal	10.64	124-19-6	-0.28 ± 1.51	2.63 ± 1.20	12.84	3.68	4.96E-08	1.282	5.79E-04
unknown 67@13.68	13.68		-0.29 ± 1.62	2.72 ± 0.85	12.84	3.68	6.21E-12	1.329	7.37E-04
unknown 98@29.25	29.25		0.21 ± 1.48	-1.99 ± 0.96	12.43	-3.64	7.26E-08	0.970	5.28E-04
3-methoxy-1-butyl acetate	9.21	4435-53-4	-0.29 ± 1.40	2.70 ± 1.39	12.3	3.62	4.21E-07	1.317	4.72E-04
methyl ester nonanoic acid	12.56	1731-84-6	-0.27 ± 1.24	2.58 ± 1.61	12.11	3.60	5.69E-06	1.259	4.05E-04
diphenylamine	34.76	122-39-4	-0.24 ± 0.94	2.22 ± 1.48	12.04	3.59	1.30E-05	1.085	1.38E-03
2-ethenyl-6-methyl-pyrazine	12.49	13925-09-2	0.16 ± 1.63	-1.51 ± 0.79	11.68	-3.55	1.66E-07	0.739	0.00E+00
4-ethyl-1,3-benzenediol	14.38	2896-60-8	-0.29 ± 1.18	2.71 ± 0.97	11.1	3.47	1.10E-09	1.325	7.83E-04
isophorone	10.85	78-59-1	-0.22 ± 1.16	2.06 ± 1.67	8.71	3.12	1.06E-04	1.005	7.21E-04
1,2-dibutyl-hydrazine	12.49	1744-71-4	-0.20 ± 0.93	1.87 ± 1.74	8.64	3.11	3.84E-04	0.916	5.29E-04
2,4-bis(1,1-dimethylethyl)-phenol	29.56	96-76-4	-0.23 ± 1.23	2.18 ± 1.46	8.57	3.10	1.28E-05	1.063	3.64E-05
5-methyl-5-(1-methylethyl)-3-heptyne-2,6-dione	11.29	63922-44-1	-0.22 ± 1.00	2.05 ± 1.51	6.66	2.73	3.93E-05	1.001	5.17E-04

Table 8 continued. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between intact male (n = 141 samples) and castrated male (n = 15 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Intact Male (N = 141)	Castrated Male (N = 15)	Raw[b]	Log_2[c]			
2-pentyl furan	7.70	3777-69-3	-0.24 ± 0.81	2.21 ± 1.16	6.49	2.70	7.54E-07	1.080	1.73E-03
unknown 176@30.01	30.01		-0.23 ± 0.93	2.21 ± 0.89	5.79	2.53	1.04E-08	1.078	4.17E-04
1-octanol	13.82	111-87-5	-0.20 ± 1.31	1.87 ± 1.42	5.71	2.51	5.18E-05	0.913	9.38E-04
2,6-bis(1,1-dimethylethyl)-4-methyl, methylcarbamate phenol	25.51	1918-11-2	-0.18 ± 0.97	1.71 ± 1.36	4.87	2.28	8.90E-05	0.837	2.75E-05
3-tert-butyl-2-pyrazolin-5-one	18.26	29211-68-5	-0.15 ± 1.32	1.43 ± 1.23	4.64	2.21	1.83E-04	0.698	7.45E-05
3-ethylcyclopentanone	9.50	10264-55-8	-0.17 ± 1.42	1.58 ± 0.95	4.39	2.13	2.16E-06	0.774	1.38E-04
1-hexanol	9.74	111-27-3	-0.17 ± 1.28	1.55 ± 1.52	4.12	2.04	6.43E-04	0.759	1.91E-04
3-octen-2-one	10.90	1669-44-9	-0.17 ± 1.37	1.64 ± 1.65	4.12	2.04	8.11E-04	0.802	3.75E-05
tri-sec-butyl ester orthoformic acid	6.02	16754-48-6	-0.17 ± 1.62	1.62 ± 1.36	4.06	2.02	1.53E-04	0.789	1.39E-04
2-methyl, 3-hydroxy-2,4,4-trimethylpentyl ester propanoic acid	20.67	74367-34-3	-0.22 ± 1.73	2.04 ± 1.28	3.94	1.98	4.42E-06	0.999	6.31E-04
benzaldehyde	13.27	100-52-7	-0.18 ± 1.70	1.70 ± 1.12	3.7	1.89	8.16E-06	0.828	1.75E-04
1-octen-3-ol	11.59	3391-86-4	-0.17 ± 1.64	1.59 ± 1.00	3.62	1.86	4.28E-06	0.777	6.20E-05

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number; VIP = Variable Importance in Projection; PLS = Partial Least Squares; RF = Random Forests

[a]All compounds were identified by spectral library search with match probability >70%

[b]Raw Fold change = abundance(condition A)/abundance(condition B)

[c]Log_2 Fold Change = log_2(abundance(condition A) – log_2(abundance(condition B))

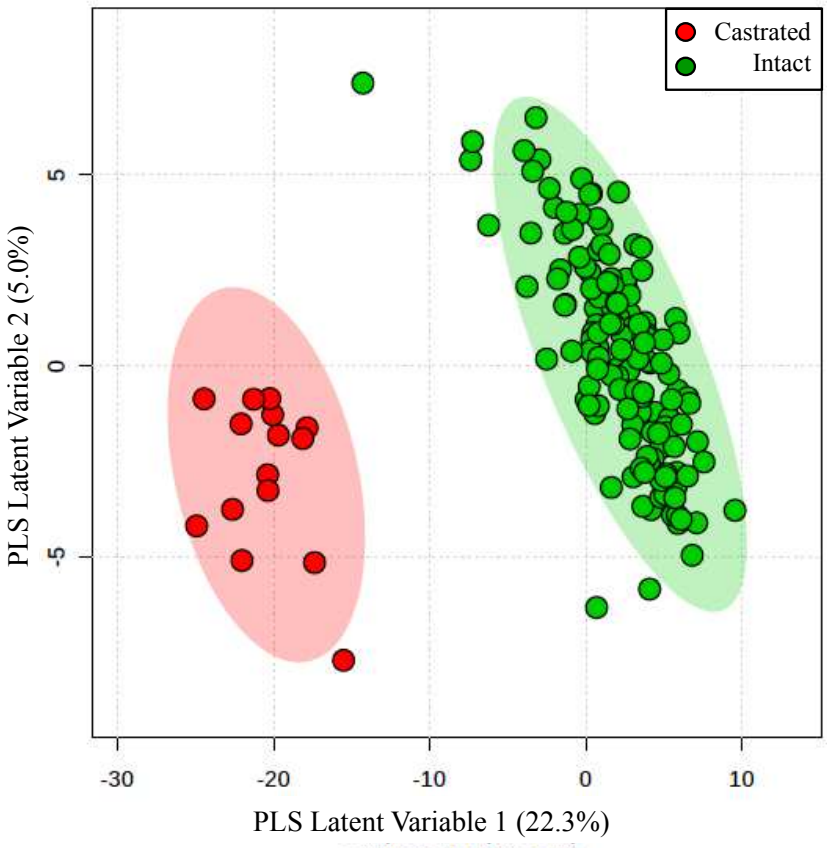


Figure 9. Scores on first 2 latent variables from partial least squares-discriminant analysis shown for intact male samples (n = 141) in green and castrated male samples (n = 15) in red. Variance explained by each latent variable is shown in brackets.

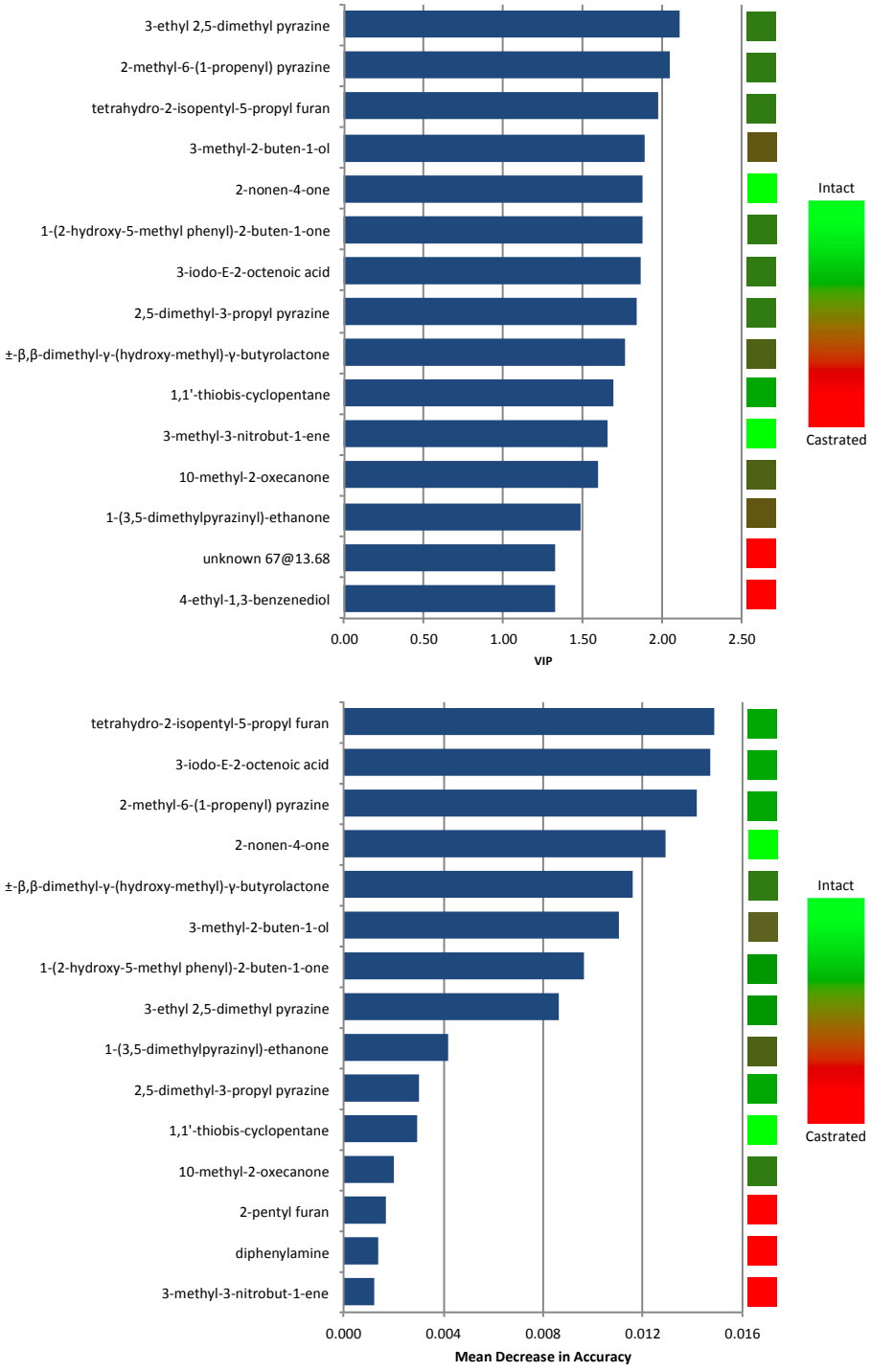


Figure 10. (a) Variable influence on projection (VIP) scores on partial least squares latent variable 1 and (b) mean decrease in classification accuracy for random forests model when each VOC is excluded for 15 influential VOCs in a comparison of intact males (n = 141 samples) and castrated males (n = 15 samples). Boxes on right indicate the relative abundance of each VOC from more abundant in intact males (green) to more abundant in castrated males (red).

Differences between paired and unpaired individuals

VOCs from paired males (SB#2660 and SB#2844) (n = 44 samples) were compared to those from unpaired males, excluding the castrated male (n = 4 individuals, 97 samples). Following peak picking and grouping procedures, 74 peaks each representing a VOC were analyzed for differential expression. Out of those 74 peaks, six urinary VOCs were found to differ significantly ($P < 0.001$ and fold change >3.0) (Table 9). Of those, five VOCs were higher in abundance in paired males than unpaired males and the other one showed higher abundance in the unpaired male samples than in those from paired males. In the PLS-DA, the 74 VOCs were reduced to five PLS latent variables that had lower discrimination ability than the other analyses ($Q^2 = 0.63$, permuted $P < 0.001$), demonstrating that these two groups are not as easily discriminated as males and females or intact and castrated males. While unpaired males were more easily classified correctly (96/97 correct) in the RF model, for paired males only 35/44 samples were correctly classified. This resulted in an OOB error = 0.0709.

Paired females (SB#2945 and SB#3184) (n = 41 samples) were compared to unpaired females SB#3231, SB#3232, SB#2845 (n = 96 samples). Following peak picking and grouping procedures, 78 peaks each representing a VOC were analyzed for differential expression. Out of those 78 peaks, seven urinary VOCs were found to differ significantly ($P < 0.001$ and fold change >3.0) (Table 10). Of those, two VOCs were higher in abundance in paired females than unpaired females and the other five showed higher abundance in the unpaired female samples than in those from paired females. In the PLS-DA, the 78 VOCs were reduced to five PLS latent variables ($Q^2 = 0.73$, permuted $P < 0.001$). While unpaired females were more easily classified correctly

(95/96 correct) in the RF model, for paired females only 33/41 samples were correctly classified. This resulted in an OOB error = 0.0657.

Analysis of breeding pair over time

One pair of maned wolves (SB#2660 and SB#2945) successfully bred and produced pups during the sampling year. The date of breeding was back calculated from the date of parturition as October 8, 2014. The temporal pattern for VOCs reported above in Table 7 (male-female differences) and Table 8 (intact male-castrated male differences) were analyzed in the successfully breeding pair to see if any of the VOCs related to the date of breeding. For this analysis, samples from male SB#2660 (n = 22) and female SB#2945 (n = 21) were processed and normalized as for the above analyses and then abundances of each VOC over time were visualized for the two wolves. There were 15 VOCs in the female that exhibited the same temporal pattern as each other with a distinctive peak on the date of breeding. These were acetophenone, ±-β,β-dimethyl-γ-(hydroxy-methyl)-γ-butyrolactone, tetrahydro-2-isopentyl-5-propyl furan, 2,5-dimethyl-3-propyl pyrazine, 2-methyl-6-(1-propenyl) pyrazine, 3-ethyl 2,5-dimethyl pyrazine, 3-iodo-E-2-octenoic acid, 2-acetyl-3,5-dimethylpyrazine, 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one, 3-methyl-2-buten-1-ol, 4-heptanone, unknown 98 at 29.25 min, tri-sec-butyl ester orthoformic acid, 3-hepten-2-one, and 1,1'-thiobis-cyclopentane (Figure 11). Two VOCs, 1,1'-thiobis-cyclopentane and 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one, showed a peak coincident with the date of breeding in both the male and the female, though 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one also showed high abundance in the male in August (Figure 12).

Table 9. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between paired male (n = 44 samples) and unpaired male (n = 97 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Paired Male (N = 44)	Unpaired Male (N = 97)	Raw[b]	Log_2[c]			
1,1'-thiobis-cyclopentane	14.48	1126-65-4	0.95 ± 1.84	-0.43 ± 1.14	9.59	3.26	2.49E-05	1.82	0.008
4-nonanone	9.46	4485-09-0	0.79 ± 1.90	-0.39 ± 1.29	6.07	2.60	8.49E-05	1.72	0.006
methyl ester benzoic acid	15.35	93-58-3	0.94 ± 1.57	-0.46 ± 1.45	5.28	2.40	2.38E-06	1.89	0.009
2-nonen-4-one	12.48	32064-72-5	0.69 ± 1.69	-0.31 ± 1.23	4.3	2.10	0.00072594	1.33	0.000
2-acetyl-6-methyl pyrazine	16.66	22047-26-3	-0.77 ± 1.22	0.38 ± 1.68	3.99	-2.00	2.55E-05	1.47	0.003
benzaldehyde	13.27	100-52-7	1.27 ± 1.19	-0.59 ± 1.59	3.95	1.98	1.57E-11	2.41	0.014

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number; VIP = Variable Importance in Projection; PLS = Partial Least Squares; RF = Random Forests

[a]All compounds were identified by spectral library search with match probability >70%

[b]Raw Fold change = abundance(condition A)/abundance(condition B)

[c]Log_2 Fold Change = log_2(abundance(condition A) – log_2(abundance(condition B))

Table 10. VOCs that differed significantly ($P < 0.001$ and fold change >3.0) between paired female (n = 41 samples) and unpaired female (n = 96 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log_2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Paired Female (N = 41)	Unpaired Female (N = 96)	Raw[b]	Log_2[c]			
1-octen-3-ol	11.59	3391-86-4	-1.00 ± 1.30	0.43 ± 1.77	37.25	-5.22	7.95E-07	2.13	0.004
benzyl methyl ketone	17.64	103-79-7	-0.88 ± 0.78	0.37 ± 1.65	17.04	-4.09	1.54E-08	1.86	0.006
1-hexanol	9.73	111-27-3	-1.44 ± 0.76	0.45 ± 1.59	15.37	-3.94	1.25E-11	2.22	0.017
3-butyl-2,5-dimethyl pyrazine	15.00	40790-29-2	0.82 ± 1.92	-0.35 ± 1.49	4.14	2.05	9.40E-04	1.74	0.006
3-ethyl-2,5-dimethyl pyrazine	11.88	13360-65-1	-0.89 ± 1.62	0.38 ± 1.61	3.34	-1.74	7.12E-05	1.89	0.013
1,3-di-tert-butylbenzene	11.25	1014-60-4	-1.08 ± 1.84	0.46 ± 1.40	3.26	-1.70	1.01E-05	2.30	0.001
styrene	8.14	100-42-5	0.84 ± 1.27	-0.36 ± 1.24	3.19	1.68	2.56E-06	1.79	0.013

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number; VIP = Variable Importance in Projection; PLS = Partial Least Squares; RF = Random Forests

[a]All compounds were identified by spectral library search with match probability >70%

[b]Raw Fold change = abundance(condition A)/abundance(condition B)

[c]Log_2 Fold Change = log_2(abundance(condition A) – log_2(abundance(condition B))

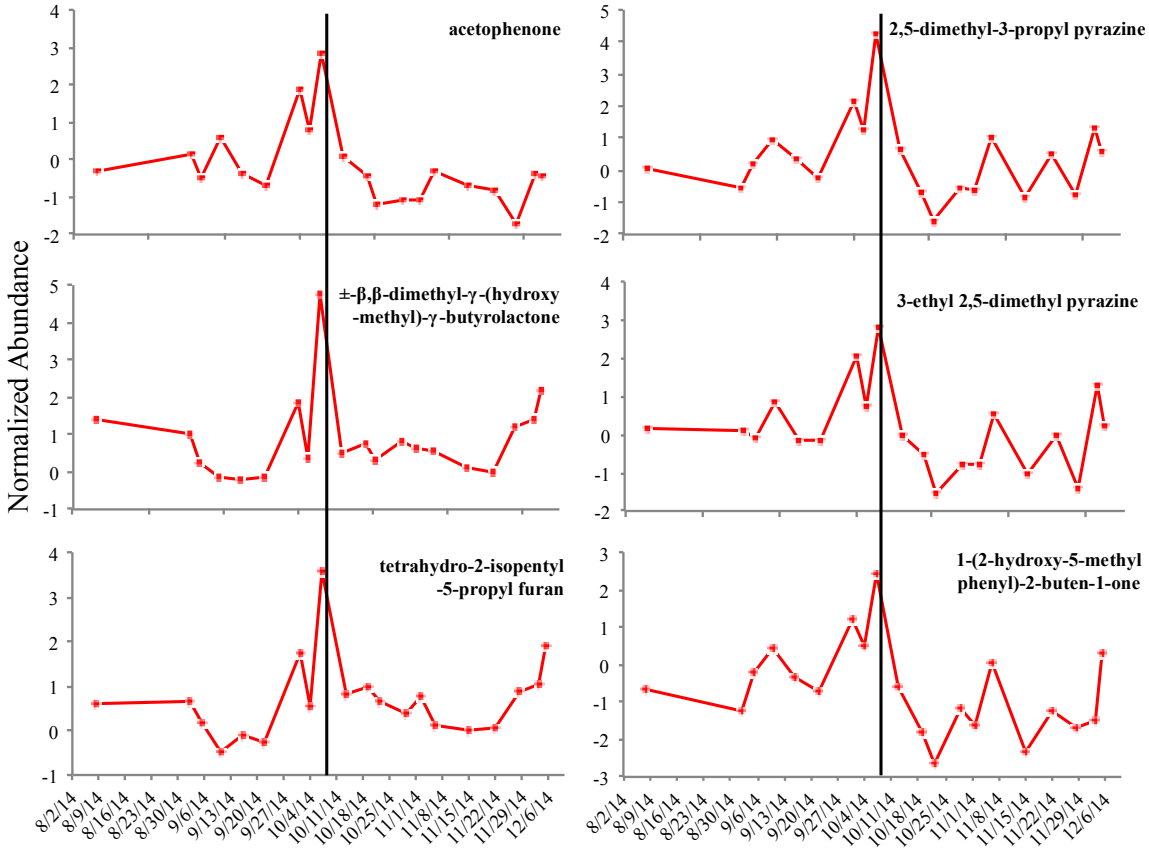


Figure 11. Six representative VOCs (out of 15) that exhibit the same temporal pattern in breeding female SB#2945 with a distinctive peak at the date of breeding (vertical line).

Figure 12. VOCs that peak in both male and female at date of breeding (vertical line).

Discussion

This study provides the most comprehensive analysis of maned wolf urinary VOCs to date, and is the first study to investigate differential expression of maned wolf urinary VOCs by sex, male reproductive status (intact or castrated), and pairing status. Eighteen VOCs differed significantly between males and females while fifty VOCs differed between intact males and a castrated male. The temporal patterns of 15 VOCs were correlated in the breeding female, with a distinctive peak on the date of breeding. Finally, there were two VOCs where both the breeding male and female showed a peak coincident with the date of copulation. Several VOCs established as important here have

been implicated in reproductive communication in other mammals. Based on their differential expression and temporal patterns, putative semiochemicals were identified as good candidates for behavioral bioassays.

Two previous studies have investigated maned wolf urinary VOCs but with limited sample size and different study goals from the present study. The aim of Goodwin et al. (2013) was to analyze 36 samples from an unspecified number of individuals to understand the role of bacteria in the changes of the most abundant VOCs over time. Childs-Sanford (2005) analyzed seven urine samples from five individuals to investigate the VOCs responsible for the strong odor of maned wolf urine and to identify sulfur based VOCs that could be responsible for the high rates of cystinuria in the species.

In light of the present findings, there is robust evidence that the urinary VOCs of maned wolves differ significantly based on sex, with the male producing higher abundances of many VOCs. Of the 18 VOCs that differed by sex, 11 were more abundant in males than females. This supports evidence in rodents (Blaustein 1981) and primates (Albone 1984; Drea 2015) that males typically produce a greater repertoire and intensity of odors. Although there are a few species that do not conform to this trend (Drea 2015), this effect is likely due to male biased sexual dimorphism in the number and size of glands (Albone 1984; Drea 2015).

The VOCs that were the strongest indicators of sex were 3-iodo-E-2-octenoic acid, 10-methyl-2-oxecanone, the unknown 98 at 29.25 min, 1,1'-thiobis-cyclopentane, and 2-nonen-4-one. These five VOCs were all higher in relative abundance in males. No literature references could be found for 3-iodo-E-2-octenoic acid or for 1,1’-thiobis-

cyclopentane. Importantly, the identifications presented here were based on spectral library searches and still need to be verified by analytical standard wherever available. 10-Methyl-2-oxecanone is the main component of the femoral gland of the Madagascan frog (*Mantidactylus femoralis*) (Poth et al. 2013), is a constituent of the odiferous defensive secretion of the eucalypt longicorn beetle, *Phoracantha synonyma* (Kitahara et al. 1983), and is produced by green mold (*Trichoderma* spp.) during periods of mycelia growth (Radványi et al. 2015). To our knowledge, this compound has not yet been reported in mammalian secretions. By contrast, 2-nonen-4-one is well known as a volatile constituent of fruits and vegetables (Buttery et al. 1970; Buttery et al. 1971). It is also a putative reproductive semiochemical found in the rutting pits of male Alaskan moose (*Alces alces gigas*) (Whittle et al. 2000) and is also found in the urine of some European badgers (*Meles meles*) (Service et al. 2001).

Striking differences were noted between intact and castrated males. The VOCs that contributed the most to the classification as intact or castrated were tentatively identified as tetrahydro-2-isopentyl-5-propyl furan, 2-methyl-6-(1-propenyl) pyrazine, and 2-nonen-4-one. However, the identities of these VOCs should still be verified via comparison to analytical standards where available. Of the top ten compounds contributing to the classification, all were more abundant in intact compared to castrated male urine samples. Tetrahydro-2-isopentyl-5-propyl furan is a VOC emitted from fresh cherries (*Prunus avium lapins*) but not found after a week of freezing (Meheriuk et al. 1995). Although specific reference was not found to tetrahydro-2-isopentyl-5-propyl furan in the mammalian semiochemical literature, furans are very common as mammalian

scent constituents. For example, 2-methyl furan is a urinary VOC for the gray wolf (Raymer et al. 1984), three furans are urinary VOCs in the African wild dog (Parker 2010), and several furans are more abundant in intact male mice (*Mus musculus*) compared to castrated males (Soini et al. 2009). 2-Methyl-6-(1-propenyl) pyrazine is one of the main volatile components of maned wolf urine (Childs-Sanford 2005; Goodwin et al. 2013) and was 124-fold less abundant in the urine of castrated males compared to intact males. 2-Nonen-4-one was also identified as important to sexual discrimination in the maned wolf. Its identity as a putative pheromone of rutting male moose (Whittle et al. 2000) was discussed above.

Even to the human nose, differences between the castrated male urine samples and those from intact males were easily detected; the urine from the castrated male did not smell strongly at all while the urine sample from intact males were quite pungent. The ten most significantly different VOCs were all more abundant in intact males as compared to castrated males, suggesting that these compounds may be dependent on testosterone. In small rodents, castrated males lose many behaviors that are associated with urinary pheromones. The ability to attract females (in mice) (Jemiolo et al. 1985; Zhang et al. 2008b), investigate the female's ano-genital region and copulate (in Syrian hamsters) (Wood and Newman 1995), accelerate puberty in females (in prairie voles (*Microtus orchogaster*)) (Lepri and Vandenbergh 1986; Petrulis 2013), and prevent implantation (in mice) (Thorpe and deCatanzaro 2012) are pheromonal effects. When castrated males are hormonally treated, these behaviors are reinstated.

Fifteen VOCs were found to be significantly different in both the analysis by sex and the analysis of intact versus castrated males. Of those 15, 11 were higher in abundance in males than females and more abundant in intact males compared to the castrated male: 2-Nonen-4-one, 1,1'-thiobis-cyclopentane, 2-ethenyl-6-methyl-pyrazine, 3-butyl-2,5-dimethyl pyrazine, 3-iodo-E-2-octenoic acid, methyl 1-methyl-2-butenyl sulfide, 3-methyl-3-nitrobut-1-ene, 3-methyl-2-butene-1-thiol, 10-methyl-2-oxecanone, 2-methyl-3-buten-2-ol, and the unknown 98 at 29.25 min. It is reasonable to hypothesize that these VOCs indicate "maleness". Behavioral bioassays with this suite of VOCs would help to further elucidate their biological roles in the maned wolf. Females should display higher behavioral interest to VOCs indicating "maleness" than to VOCs that are present in maned wolf urine but are not thought to convey a chemical message.

The suite of VOCs that peaked near the date of copulation in the breeding female (Figure 11) should also be used in bioassays with males. If males exhibit higher behavioral interest in these VOCs as compared with other maned wolf urinary VOCs that are not believed to contain reproductive messages that would lend support to the hypothesis of these VOCs being important to reproductive communication. Among these 15 VOCs were acetophenone, 2,5-dimethyl-3-propyl pyrazine, 3-ethyl 2,5-dimethyl pyrazine, 4-heptanone, and 3-hepten-2-one.

Acetophenenone is a common constituent of mammalian urine from Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*) (Goodwin et al. 2012) to mice (Soini et al. 2009). It is also found in the urine of several canids including the maned wolf (Jorgensen et al. 1978; Raymer et al. 1986; Schultz et al. 1988; Parker 2010;

Goodwin et al. 2013). In the only study investigating sex differences, acetophenone is indicative of female gray wolves (Raymer et al. 1986). Levels increase in males after castration, and levels fall with addition of testosterone to these males (Raymer et al. 1986). 3-Ethyl-2,5-dimethyl pyrazine and 2,5-dimethyl-3-propyl pyrazine are pheromones for several species of Hymenoptera (Morgan et al. 1999; Hölldobler et al. 2001). Although they were both previously identified in maned wolf urine (Goodwin et al. 2013), differential expression by sex has not yet been investigated. 4-Heptanone is found in urine across a wide variety of mammals and the abundance differs between the sexes for some species. In the cheetah (*Acinonyx jubatus*) this compound is only found in female urine (Burger et al. 2006), while in the ferret (*Mustela furo*) 4-heptanone is more abundant in males than in females (Zhang et al. 2005), and in the rat (*Rattus norvegicus*) this compound is only found in male urine (Zhang et al. 2008b). 4-Heptanone also occurs in the urine of African elephant females during both surges of the reproductive hormone, luteinizing hormone (Goodwin et al. 2005). 3-Hepten-2-one occurs in the urine of both sexes of *Phodopus* hamsters (Soini et al. 2005) and both sexes of the cheetah (Burger et al. 2006). To our knowledge, this VOC has not yet been reported in canid urine.

This study provides an excellent foundation for selecting VOCs that may play a role in estrus or ovulation induction in the maned wolf. 1,1'-thiobis-cyclopentane and 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one are the strongest candidates for bioassay testing. Unfortunately no references to either of these compounds could be found in the literature. In the present study 1,1'-thiobis-cyclopentane had an 8-fold higher relative abundance in males compared to females and over a 200-fold increase in relative

abundance in intact males compared to the castrated male. Further, this compound peaked on the day of breeding for both individuals that successfully bred during the study period. 1-(2-Hydroxy-5-methylphenyl)-2-buten-1-one showed a 77-fold increase in abundance in intact males compared to the castrated male and showed a similar temporal profile to 1,1'-thiobis-cyclopentane where the abundance peaked in both sexes of the breeding pair on the day of breeding. Behavioral bioassays should show high behavioral interest in these scents and if the compounds are responsible for estrus or ovulation induction, endocrine profiles of singly housed females should demonstrate ovarian activity indicative of estrus (sharp peak in estrogen levels) or ovulation (prolonged elevation of progestagens).

Building on this work, future analytical chemistry studies should strive to collect samples from several breeding pairs with increased frequency around the time of breeding to better ascertain which VOCs may play a role in maned wolf reproduction. However, this is challenging. Because the species is an induced ovulator, estrus is a 2 – 5 day window within several months. Though mean breeding season is November (Rodden et al. 1996), individuals have been observed breeding as early as September and as late as April (Rodden et al. 2007). Although urine collections for this study were all non-invasive and many institutions had few issues collecting regular samples, several institutions found the time demands of the collection protocol too cumbersome and declined to participate or agreed to participate but were only able to collect a few samples over the course of the study.

These results add valuable information to the growing body of knowledge of mammalian semiochemistry. The maned wolf is only the fifth species (out of 36) within

Canidae to be investigated for urinary VOCs. Few of the existing canid studies have attempted to analyze differential expression by sex (excepting Raymer et al. 1984; Raymer et al. 1986; Parker 2010; Apps et al. 2012), and none have done so in the maned wolf. This work lays an important foundation for semiochemical discovery in this species and establishes a robust data analysis pipeline that can be widely adopted to improve differential analyses in any species to answer an infinite number of biologically relevant questions.

CHAPTER 4: HORMONAL AND BEHAVIORAL RESPONSES OF GROUP-HOUSED FEMALE MANED WOLVES TO MALE BREEDING SEASON URINE

Abstract

The maned wolf (*Chrysocyon brachyurus*) is an induced ovulator. Though the mechanism of ovulation induction remains unknown, it is suspected to be a urinary chemical signal. This study assesses whether presenting male urine to females can prompt ovarian activity and behavioral interest without the physical presence of a male. Secondarily, the effect of group housing females on reproductive hormones and behaviors was investigated. Seven female maned wolves were used in this study; three treatment females (dam and two daughters housed together) were exposed to male urine for 30 days while four females (two sister pairs each housed together) formed the control group and were not exposed to urine stimuli. Fecal samples were collected 3 – 4 times weekly from seven adult female maned wolves during September - December of 2014 and were processed to assess metabolites of gonadal hormones. The male urine was collected from one singly housed adult male during breeding season and was frozen until use. Behavioral observations were conducted for the treatment females for 60 min daily in pre-, during, and post-treatment periods, each lasting a month. None of the seven females showed hormone levels consistent with ovulation, but some ovarian activity in the treatment dam was observed. Compared with controls, treatment females showed significantly higher estrogen metabolite levels (58.51 ± 2.10 v. 49.32 ± 1.34 ng/g dried

feces) but lower progestagen metabolite levels (0.96 ± 0.10 v. 1.69 ± 0.32 μg/g dried feces). Behavioral interest was higher when urine was present, averaging 6.15 sec sniffing per hour, compared with 3.35 sec/h during the pre-exposure period. All of the instances of urine scent marking (n = 26) were by the dam. In two of the three groups, one female had higher reproductive hormone concentrations than conspecifics. Co-housed females also engaged in agonistic encounters on average 8.5 times per hour and the female with the highest reproductive hormone levels was the female dominant in behavioral interactions. Overall, the results from this preliminary study support the hypothesis that maned wolf urine contains reproductive semiochemicals that affect female ovarian hormones. Specifically (1) daily exposure to male urine prompted an ovarian response in some individuals, though it was not substantial enough to induce ovulation, (2) reproductive suppression may occur in this species, and (3) co-housing females leads to social tension. Future studies comparing responses of females exposed to urine from paired and unpaired males and with and without visual access to a male are recommended to further elucidate the mechanism of ovulation induction in the maned wolf. An expanded study of co-housed females is also warranted to further investigate reproductive suppression in this species.

Introduction

The maned wolf (*Chrysocyon brachyurus*) is a unique member of Canidae (the dog family), endemic to tropical grasslands of South America (Dietz 1985). The species is listed by the IUCN RedList as "Near Threatened", with an estimated wild population of around 17,000 (Paula and DeMatteo 2015). The maned wolf is a seasonal monoestrous

breeder meaning that females cycle just once per year during the breeding season (Asa and Valdespino 1998; Sillero-Zubiri et al. 2004). Maned wolves are solitary, with juveniles dispersing from their mother before two years of age (Brady and Ditton 1979; Dietz 1984; Bestelmeyer 2000; Emmons 2012). Prime reproductive years are ages 3 – 8, though females aged 2 – 12 have successfully produced offspring (Rodden et al. 2007; Songsasen and Rodden 2010).

Intriguingly, the maned wolf seems to be an induced ovulator, meaning that females ovulate only in the presence of a male (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). The only other presumed induced ovulating canid is the Channel Island fox (*Urocyon littoralis*) (Asa et al. 2007). The domestic dog (*Canis familiaris*) (Concannon et al. 2009; Concannon 2011), gray wolf (*Canis lupus*) (Seal et al. 1979; Asa et al. 2006), red wolf (*Canis rufus*) (Walker et al. 2002), coyote (*Canis latrans*) (Carlson and Gese 2008), African wild dog (*Lycaon pictus*) (Monfort et al. 1997; Van der Weyde et al. 2015), bush dog (*Speothos venaticus*) (DeMatteo et al. 2006), red fox (*Vulpes vulpes*) (Mondain-Monval et al. 1977; Maurel et al. 1984), and arctic fox (*Alopex lagopus*) (Möller 1973) are spontaneous ovulators and other species of canid have yet to be investigated for this trait.

For female maned wolves paired with a male, researchers have determined that patterns of gonadal hormone secretion (Wasser et al. 1995; Velloso et al. 1998; Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a) are similar to patterns seen in the domestic dog (Wasser et al. 1995; Velloso et al. 1998; Songsasen et al. 2006; de Gier et al. 2006; Concannon 2011; Reiter 2012; Johnson et al. 2014a) and most other wild

canids (Monfort et al. 1997; Walker et al. 2002; DeMatteo et al. 2006; Asa et al. 2006; Asa et al. 2007). Estrus, the period of reproductive receptivity, lasts between 1 – 10 days (Songsasen et al. 2006; Rodden et al. 2007). It is characterized by an estrogen peak followed 16 days later by a rise in progestagen (Songsasen et al. 2006; Songsasen et al. 2014). Exact timing of ovulation is only known via measuring luteinizing hormone and in the domestic dog ovulation occurs two days after the estrogen peak (de Gier et al. 2006; Concannon 2011). Copulation begins 2 – 6 days after the estrogen peak, and can occur up to nine days following the peak (Songsasen et al. 2006; Songsasen et al. 2014). On the day of successful copulation, estrogen levels decrease back to baseline (Songsasen et al. 2006; Songsasen et al. 2014). Regardless of pregnancy status, paired females show a ~65 day rise in progestagens above baseline (Songsasen et al. 2006). Pregnant females show higher progestagen levels than non-pregnant females during this phase (Reiter 2012).

Because maned wolves are induced ovulators, females housed without a male show baseline progestagen levels through the entire breeding season, indicating a lack of ovulation (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). In 2004, a pair that had been separated for the duration of the normal breeding season to prevent pregnancy copulated shortly after being reintroduced in April and gave birth more than two months outside the typical breeding and birthing season (Rodden et al. 2007). This suggests that the presence of a male strongly influences the timing of ovulation. In 2009, a female housed singly but sharing a fence line with a male ovulated (Johnson et al. 2014a). In this instance the female had visual access to the male as well as the ability to contact his urine scent marks deposited on the shared fence line. In the same reproductive season, several

other females housed diagonally from a male or farther from males failed to ovulate (Songsasen, pers. comm.), suggesting that visual stimulus is not sufficient and an olfactory mechanism underlies this phenomenon. Finally, a female paired with a castrated male showed baseline ovarian hormone concentrations (Jones, unpublished data) suggesting that the mechanism of ovulation induction is correlated to testosterone levels.

Chemical communication is known to play an important role in mammalian behavior and reproductive processes for many species (Müller-Schwarze 2006; Dehnhard 2011). For the Canidae, urine is considered to be a more important source of scent signaling than feces (Gese and Ruff 1997; Parker 2010; Apps et al. 2012; Jordan et al. 2013). Urine scent marking, but not defecation, increases in frequency during the breeding season for several canid species including the maned wolf (Asa et al. 1984b; Hradecky 1985; Asa et al. 1990; Rodden et al. 1996; Gese and Ruff 1997; Pal 2003; Parker 2010; Jackson et al. 2012; Jordan et al. 2013). Maned wolf urine, like that of other canids, has a very distinctive pungent odor with scent marks remaining detectable to humans for several weeks (Emmons 2012; Goodwin et al. 2013). Rates of scent marking do not differ between males and female maned wolves (Rodden et al. 1996). Within males, the frequency of urine scent marking is highest during proestrus compared to other stages of the reproductive cycle and is significantly higher in successful breeders when compared to unsuccessful breeders (Rodden et al. 1996). Similarly, successfully breeding females scent mark significantly more than those who are unsuccessful (Rodden et al. 1996). Urine scent marking is clearly important to maned wolf reproduction, hypothetically because urine contains chemical signals important to reproduction.

This study tested whether frozen-thawed male maned wolf urine collected during breeding season provides a sufficient chemical cue to promote ovarian activity and sustained behavioral interest in the female maned wolf without the physical presence of a male. I hypothesized that if male breeding season urine contains bioactive compounds, females exposed to breeding season urine should exhibit high, consistent behavioral interest in the stimuli and signs of ovarian activity characterized by elevated estrogen levels or by ovulation indicated by elevated progestagen levels. Determining that the mechanism of ovulation induction in the maned wolf is via olfactory communication would be significant because there are few induced ovulating carnivores, and of those, none are known to ovulate in response to olfactory cues (Larivière and Ferguson 2003). We speculate that the maned wolf exhibits a novel ovulation mechanism in carnivore reproduction via urinary chemical signals. Defining novel reproductive mechanisms has been cited as the highest priority of wildlife research today (Pukazhenthi and Wildt 2004; Comizzoli et al. 2009). Breeding programs could exploit knowledge about ovulation induction in this species to better time assisted reproductive technologies or to improve success of natural breeding in this threatened species.

Secondarily, because maned wolves are typically solitary, this study provided an excellent opportunity to investigate the effect of group housing on the female reproductive cycle. Studies have examined group housing and reproduction in solitary felids, but reproductive hormone data for co-housed solitary canids has not yet been investigated. Female cheetahs (*Acinonyx jubatus*) are solitary. While group housing highly related females does not alter reproductive hormone output (Koester 2014), if

unrelated females are housed together, ovarian activity decreases (Wielebnowski et al. 2002). In small felids chronic stress and decreased reproductive output are associated with social housing (Mellen et al. 1998; Swanson et al. 2003; Price and Stoinski 2007). There are multiple species of social canids where only one female of a social group breeds and the others experience reproductive suppression, characterized by lower progestagen output and a failure to ovulate (Creel and Creel 1991; Creel and MacDonald 1995; Creel et al. 1997; Kleiman 2011). Thus, we expected social housing in the maned wolf to lead to agonistic encounters between females. Furthermore, we expected that socially dominant females would show higher ovarian hormone levels compared to others. Because co-housing of maned wolf sibling pairs and family groups is a common practice in North American zoological institutions, this data will provide important insights into the effects of co-housing a solitary canid species how best to manage co-housed groups.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Smithsonian National Zoo and Conservation Biology Institute or its equivalent at each participating institution. Seven female maned wolves were used for this study from September 1, 2014 to December 31, 2014 (Table 11). Three females (Stud Book (SB) #2539, SB#3253, SB#3254) housed at the Smithsonian Conservation Biology Institute (SCBI, Front Royal, VA) as a family group were assigned to the treatment group and exposed to the male urine stimuli. This group was housed in

an enclosure totaling ~1660 m^2 with access to climate-controlled buildings (16 m^2). During the periods of behavioral observation the group was confined to a portion of their enclosure ~450 m^2 (Figure 13). Four females comprised the control group and were not exposed to male urine stimuli. Two sisters (SB#3231, SB#3232) housed together at Beardsley Zoo (Bridgeport, CT) had access to an outdoor enclosure (~760 m^2) and heated dens. A second pair of sisters (SB#3175, SB#3177) was housed together at the Endangered Wolf Center (EWC, Eureka, MO) in an outdoor enclosure (~2000 m^2) with access to a heated den. All wolves were fed a diet of custom maned wolf kibble (Mazuri, Land O'Lakes, Inc., Richmond, IN) supplemented with seasonal fruits and whole prey items (mice and guinea pigs). Water was available *ad libitum*. None of the females were housed in proximity to males for the duration of the study.

Table 11. Female maned wolves that supplied fecal samples and served as subjects for behavioral observations (treatment only).

SB#[a]	Age (years)	Experiment Group	Institution[b]	Housing	Total Enclosure Area (m^2)	Proven Breeder
2539	11	Treatment	SCBI	Family group	1660	Yes
3253	2	Treatment	SCBI	Family group	1660	No
3254	2	Treatment	SCBI	Family group	1660	No
3231	3	Control	Beardsley	Sister pair	760	No
3232	3	Control	Beardsley	Sister pair	760	No
3175	4	Control	EWC	Sister pair	2000	No
3177	4	Control	EWC	Sister pair	2000	No

[a]SB# = Studbook number. Association of Zoos and Aquarium reference number of individual pedigree and demographic history.
[b]SCBI = Smithsonian Conservation Biology Institute, Front Royal, VA; Beardsley = Beardsley Zoo, Bridgeport, CT; EWC = Endangered Wolf Center, Eureka, MO

Urine was pooled from 22 samples collected at SCBI from a singly housed male (SB#2844) during peak breeding season, October and November of 2010 (age four) and

2011 (age five). Although this male did not breed, semen collections in these years showed that he produced viable sperm (Songsasen, pers. comm.). SB#2844 is the uncle of SB#2539 and therefore, the great uncle of SB#3253 and SB#3254. The urine samples were frozen at -20°C upon collection and remained frozen until time of use except for a brief period of defrosting to allow for pooling and portioning the 6 mL samples necessary for this study.

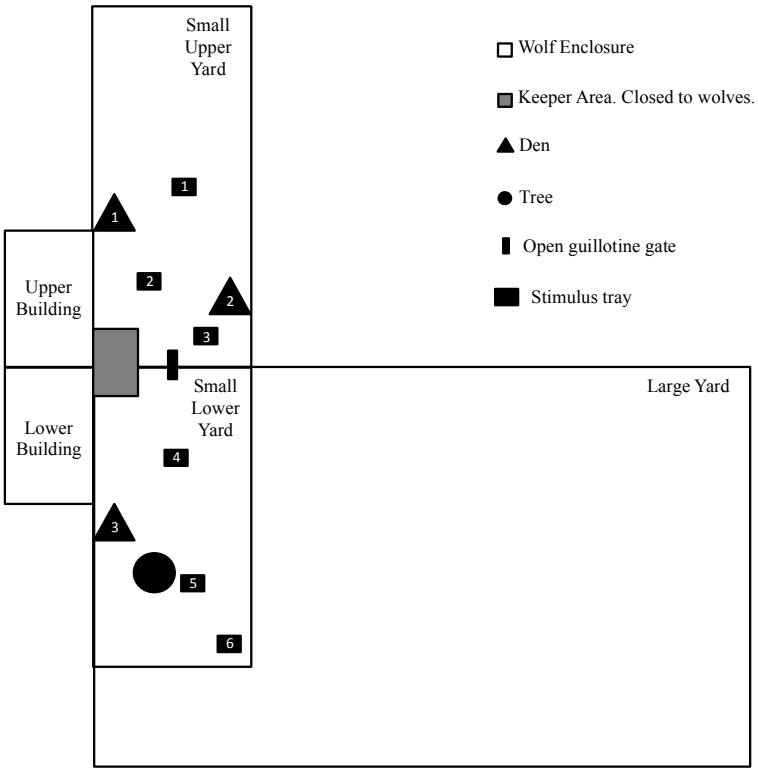


Figure 13. Layout of wolf enclosure at the Smithsonian Conservation Biology Institute denoting position of dens (numbered triangles), stimuli trays (numbered rectangles), the tree (circle), and the gate between yards (vertical rectangle). Wolves were maintained in small yards during behavioral observations.

Endocrinology methods

Fecal samples (10 g) were collected from all seven females 3 – 5 times per week for the duration of the study. Because individuals were housed socially, food dye was added to the wolf's diet in order to distinguish samples from each individual. Fresh samples (within 12 hours of defecation) were collected and stored at -20°C until hormonal analysis (Brown 2008). Extraction of estrogen and progestagen metabolites from fecal samples was performed as previously described (Wasser et al. 1995; Pukazhenthi and Wildt 2004; Songsasen et al. 2006; Brown 2008; Reiter 2012; Johnson et al. 2014a). After extraction, each sample was re-suspended in 1 mL dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.2 M NaCl, pH 7.0) and then stored at -20°C until analysis. The mean extraction efficiency for this study was 73.7%.

Fecal estrogen and progestagen metabolites were quantified using single antibody enzyme immunoassays, as previously described (Velloso et al. 1998; Songsasen et al. 2006). Fecal extracts were diluted in appropriate buffer (estrogens, 1:10 – 1:40; progestagens, 1:50 – 1:5000). Estrone conjugate (polyclonal antibody R 522-2) and pregnane (monoclonal antibody CL425) antibodies were obtained from the University of California, Davis, CA. Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the appropriate standard. Interassay coefficients of variation for two internal controls were 7.6% (mean binding: 40.2%) and 5.8% (mean binding: 72.2%) for estrogen (n = 16 assays) and 11.9% (mean binding: 32.8%) and 8.9% (mean binding: 71.6%) for progestagen (n = 27 assays). Intra-assay coefficients of variation for both assays were <10%.

Behavioral methods

For the three treatment females, six metal stimuli trays (33 x 23 x 8 cm) were installed throughout the small upper and lower yards of the enclosure (Figure 13). During the months of September (pre-treatment) and November (post-treatment), 500 g of washed sand (Quikrete, Clinton, MD) was added to each tray and replaced daily (Raymer 1984). For October (treatment period), 6 mL of male urine was added to the sand daily.

Behavioral observations were conducted for the three treatment females. Three video cameras (Night Owl SP LLC, Walpole, MA) were installed throughout the shared enclosure of the three treatment females to allow full coverage of the outdoor area. Behavior was recorded each day from September 1, 2014 through November 30, 2014. Observation sessions were 60 min beginning when the animal keeper replaced the stimuli trays, between 9AM – 12PM.

Video footage was reviewed to ensure that each session was at least 55 min in duration (i.e., cameras recorded at least 55 min after stimuli trays were changed) and that the gates to neighboring yards were closed to ensure that cameras would fully cover the observation area. Sessions meeting these criteria (n = 44) were then coded using scan sampling (Martin and Bateson 2007) to record the duration of every interaction with the stimuli trays and every instance of scent marking (Appendix 5: Maned wolf ethogram). Scent marking was defined as urination on any surface other than the ground. A bout of interaction with the tray began when a wolf was within two body lengths of the stimulus tray. The only behavior that was observed in response to the stimuli trays was "sniff object" and this was defined as extending the nose towards the stimulus tray. Daily

duration of interactions with the stimuli trays and duration of each bout of interaction were calculated for each session.

Out of the 44 sessions meeting the criteria, the first five dates of each month were also coded using focal animal observations (Martin and Bateson 2007) to record each individual's state behaviors (i.e., walking, running, lying down, etc.) each minute, proximity to the closest stimulus tray, and proximity to the other two wolves each minute (Appendix 5: Maned wolf ethogram). For these 15 sessions, descriptions of all agonistic encounters between the wolves were documented (Rodden et al. 1996). Any occurrence of an individual reacting to the presence of conspecific was considered an encounter. Encounters were described with the proximity of the wolves to one another, and with the body postures of each wolf throughout the encounter (Appendix 5: Maned wolf ethogram). Because higher posture is associated with dominance in the maned wolf (Biben 1983), relative body postures were noted throughout the encounter.

Data analysis

Mean and baseline fecal hormone metabolite levels were calculated for each wolf and each hormone. The baseline represents the basal hormone metabolite excretion for an individual excluding the rise in hormone output associated with reproduction (Scarlata et al. 2013). To calculate a baseline, data points above a given standard deviation (SD) threshold were removed until no additional data points exceeded the threshold (Brown et al. 1994; Brown et al. 2001). The baseline was calculated as the mean of the remaining points. Thresholds of 1.5, 2.0, and 2.5 SDs were considered. Two SD was chosen because this value produced a baseline that included the greatest average number of data points

across all individuals and hormones, while creating reasonably normal distributions within each individual's data set (Brown et al. 2016). Because analyses based on mean hormone level were the same in every case to those using the baseline hormone level, only the results of the analyses of the mean hormone levels were reported.

Hormone profiles of all females were aligned by the first day of exposure to male urine (October 1, 2014). Ovulation was defined by a rise in progestagen level 2 SD above baseline for at least three consecutive days (Johnson et al. 2014a) and a mean progestagen metabolite concentration during the luteal phase above 10 μg/g feces. This value was selected based on findings in Songsasen et al. (2006) that 10 μg/g feces during the luteal phase was a definitive separator between singly housed females who failed to ovulate and paired females who were suspected to have ovulated. The last three dates measured for wolf #3175 showed abnormally high values of fecal progestagen metabolites (30 times higher than her other values). This individual died five months after this study concluded. Necropsy reports showed evidence of uterine pathology that could have affected gonadal hormone concentration. Therefore, data from these three dates were removed as outliers prior to statistical analysis. Hormone concentrations were $\log_{10}$ transformed prior to statistical analysis to better meet the assumption of normality. Comparisons of estrogen and progestagen concentrations among groups were performed using the Student's T-test or ANOVA followed by Tukey's multiple comparison tests. Because of high variability between individuals, each wolf was analyzed individually for differences by month using ANOVA followed by Tukey's multiple comparison tests.

For analysis of the behavior data, state behaviors were clustered into “inactive”, which included “not visible” and “inactive lying down” and “active”, which included “alert”, “sitting”, “standing”, “walking”, “running”, and “other” (Appendix 5: Maned wolf ethogram). State behaviors and time spent in proximity to trays and other wolves were treated as proportions; the number of minutes spent in the state was divided by the total number of minutes in the session. Chi square tests were used to assess differences in proportions across individuals and months. Continuous variables were assessed for normality. Due to deviations, Kruskal-Wallis tests were performed to analyze behavioral differences across individuals and months.

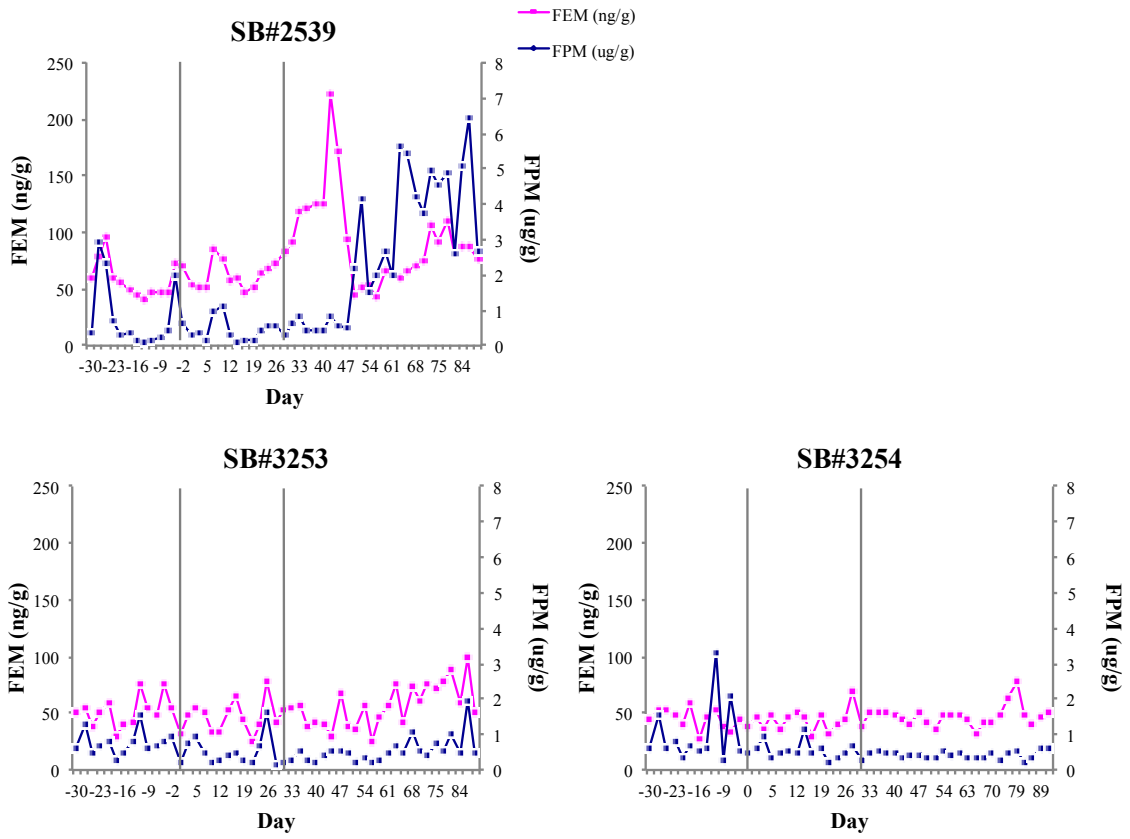
All analyses were performed in R (R Core Team. 2013. R: a language and environment for statistical computing). Results were considered significant at $P \leq 0.05$ and were reported as mean ± standard error of mean.

Results

Hormonal response

In total, 345 fecal samples from seven individuals were processed to determine fecal estrogen metabolite concentrations (FEM) and fecal progestagen metabolite concentrations (FPM). Each individual was sampled on average 49.3 ± 4.40 days. Using data from all months, mean FEM was higher in the treatment females (58.51 ± 2.10 ng/g dried feces) than in controls females (49.07 ± 1.36 ng/g dried feces, $t_{325.6} = 4.33$, $P < 0.0001$) (Figure 14, Figure 15). This effect was due to high FEM values for SB#2539 (76.57 ± 4.71 ng/g dried feces), much higher than all other females ($F_{6,334} = 34.22$, $P < 0.0001$) (Figure 14, Figure 15). For FPM, treatment females showed lower mean levels

(0.96 ± 0.10 µg/g dried feces) than control individuals (1.15 ± 0.06 µg/g dried feces, $t_{274.6}$ = -4.95, $P < 0.0001$) (Figure 14, Figure 15) though treatment female SB#2539 again had the highest mean value of any of the females (1.68 ± 0.25 µg/g dried feces, $F_{6,334} = 13.29$, $P < 0.0001$).



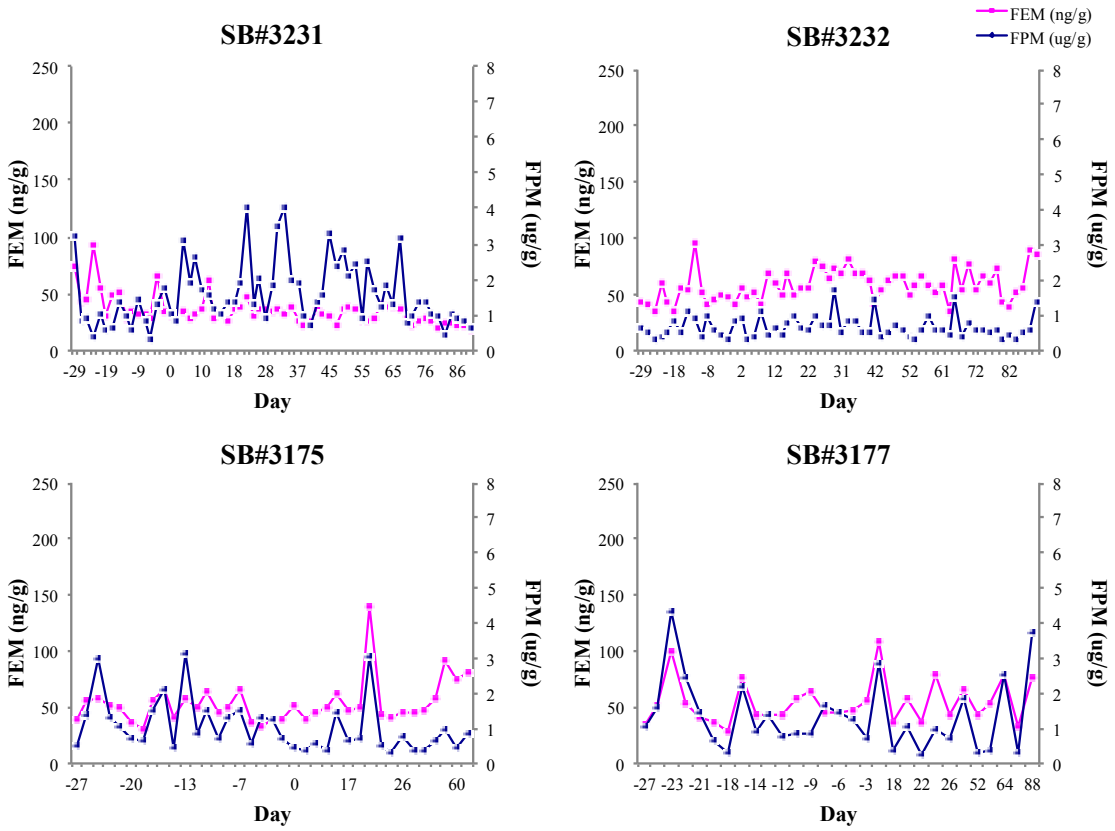


Figure 14. Fecal estrogen metabolites (FEM) and fecal progestagen metabolites (FPM) for n = 3 maned wolf females (SB#2539, 3253, 3254) housed as a family group exposed to 36 mL male urine daily during October (marked by vertical lines). SB#2539 is the dam and SB#3253 and SB#3254 are the daughters and n = 4 maned wolf females housed as two sister pairs (SB#3231 and 3232) and (SB#3175 and 3177) not exposed to male urine.

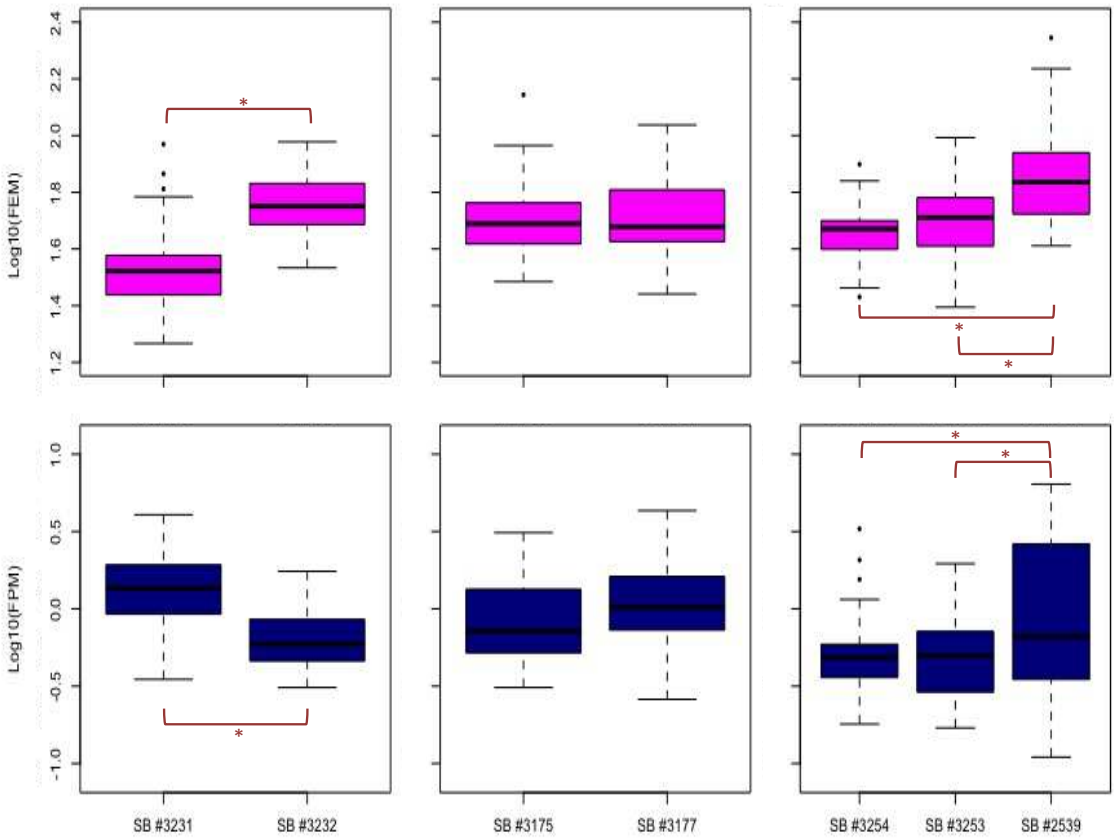


Figure 15. Mean log_{10} transformed concentrations of fecal estrogen metabolites (FEM) and fecal progestagen metabolites (FPM) for female maned wolves housed as three groups.

Considering only the treatment females, FEM levels were higher in SB#2539 (76.57 ± 4.71 ng/g dried feces) than for her daughters who did not differ from each other (52.91 ± 2.34 and 45.80 ± 1.34 ng/g dried feces) ($F_{2,149} = 31.62$, $P < 0.001$) (Figure 14, Figure 15). The same trend was observed for FPM. SB#2539 showed an average FPM level of 1.68 ± 0.25 µg/g dried feces compared with SB#3253 (0.59 ± 0.05 µg/g dried feces), and SB#3254 (0.60 ± 0.07 µg/g dried feces) ($F_{2,149} = 8.04$, $P < 0.001$) (Figure 14, Figure 15). For one pair of control females (SB#3231 and SB#3232), SB#3231 showed lower FEM than her sister (35.25 ± 1.62 v. 58.25 ± 1.80, $t_{116.5} = -10.62$, $P < 0.0001$) but

higher FPM (1.56 ± 0.11 v. 0.68 ± 0.04, $t_{114.8} = 8.70$, $P < 0.0001$). The other pair (SB#3175 and SB#3177) did not show differences in either hormone (FEM: $P = 0.86$, FPM: $P = 0.28$) (Figure 14, Figure 15).

Table 12. Fecal estrogen and progestagen metabolite concentrations for seven female maned wolves exposed to male urine during October (Trt = treatment) or not exposed (Ctrl = control).

SB#	Group	September	October	November	December	*F*	Sig.
FEM (ng/g)							
2539	Trt	59.51 ± 4.51	65.62 ± 3.84	103.34 ± 15.95	81.05 ± 4.69	4.84	$P = 0.005$
3253	Trt	51.76 ± 3.73	46.8 ± 3.77	44.66 ± 3.62	69.55 ± 4.61	6.75	$P < 0.001$
3254	Trt	45.15 ± 2.71	43.55 ± 2.53	46.01 ± 1.55	49.05 ± 3.52	0.76	$P = 0.524$
3231	Ctrl	46.41 ± 4.88	35.59 ± 1.97	31.15 ± 1.45	27.94 ± 1.88	8.94	$P < 0.001$
3232	Ctrl	50.19 ± 3.98	58.63 ± 2.95	61.76 ± 2.47	61.87 ± 4.34	2.91	$P = 0.042$
3175	Ctrl	48.32 ± 2.53	55.75 ± 8.56	63.43 ± 8.94	80.64 ± 0.00	2.07	$P = 0.123$
3177	Ctrl	51.04 ± 4.22	60.43 ± 11.63	54.69 ± 6.76	63.03 ± 14.83	0.40	$P = 0.754$
FPM (ug/g)							
2539	Trt	0.84 ± 0.26	0.44 ± 0.08	1.38 ± 0.34	4.34 ± 0.39	25.55	$P < 0.001$
3253	Trt	0.78 ± 0.10	0.49 ± 0.10	0.35 ± 0.04	0.74 ± 0.13	7.24	$P < 0.001$
3254	Trt	1.00 ± 0.25	0.53 ± 0.06	0.44 ± 0.03	0.43 ± 0.04	4.55	$P = 0.007$
3231	Ctrl	1.10 ± 0.18	1.76 ± 0.20	2.14 ± 0.24	1.19 ± 0.16	7.35	$P < 0.001$
3232	Ctrl	0.62 ± 0.07	0.77 ± 0.09	0.66 ± 0.08	0.64 ± 0.09	0.85	$P = 0.473$
3175	Ctrl	1.28 ± 0.17	0.84 ± 0.24	0.57 ± 0.11	0.87 ± 0.00	3.24	$P = 0.034$
3177	Ctrl	1.41 ± 0.23	1.05 ± 0.38	0.84 ± 0.51	2.21 ± 1.00	1.20	$P = 0.331$

For the treatment dam SB#2539, FEM levels were higher in November (103.33 ± 15.95 ng/g dried feces) than in any other month, while her FPMs peaked in December (Table 12). Treatment daughter SB#3253 had slightly higher levels of FEM in December than in other months and her FPM showed high concentrations in September and December (Table 12). Treatment daughter SB#3254 on the other hand showed no differences in FEM levels by month, and the highest concentration of FPM occurred during September (Table 12).

For the control females together, there were no differences by month for FEM ($F_{3,188} = 0.35$, $P = 0.79$) or FPM ($F_{3,188} = 0.54$, $P = 0.65$). Control female SB#3231 showed the highest FEM in September (46.41 ± 4.88 ng/g dried feces) compared to other months but higher FPM during October and November (Table 12). Her sister, SB#3232, had slightly lower levels of FEM during September as compared to other months, but showed no differences by month for FPM. Finally, control females SB#3175 and SB#3177 showed no differences by month for FEM and SB#3175 had marginally higher FPM in September as compared with other months (Table 12).

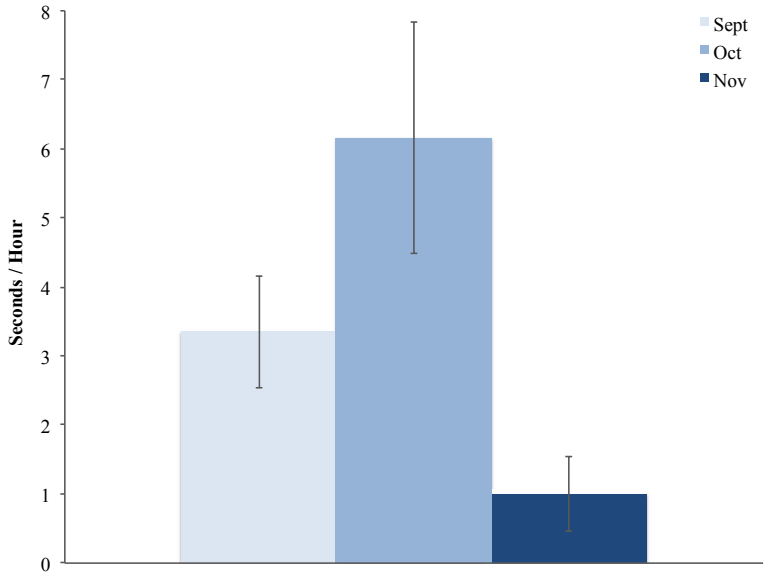


Figure 16. Average sniffing duration for three maned wolves during pre-treatment (Sept), treatment (Oct), and post-treatment (Nov) sniffing stimuli trays containing male breeding season urine.

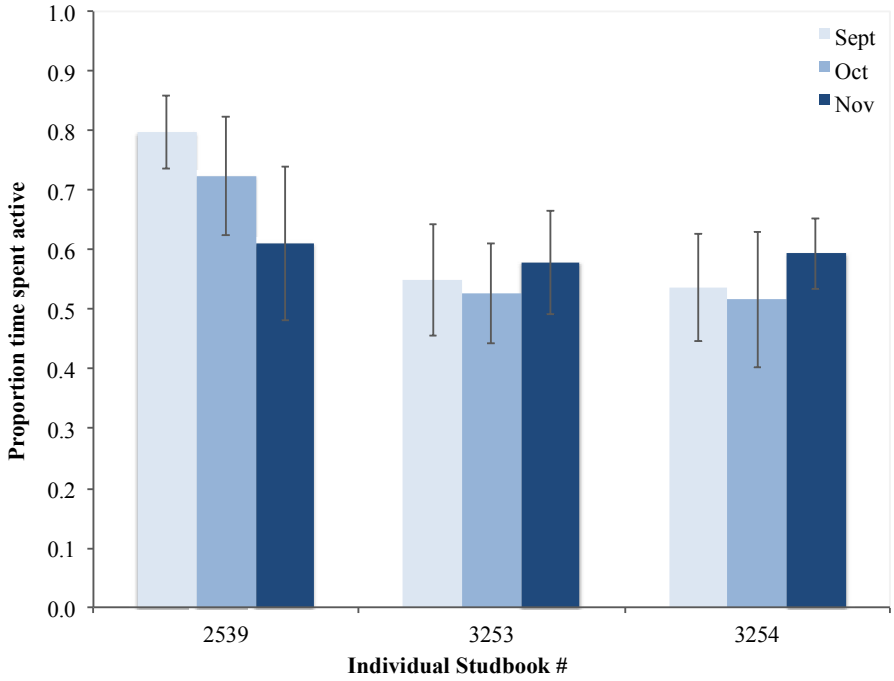


Figure 17. Proportion of time spent active for three maned wolves during pre-treatment (Sept), treatment (Oct), and post-treatment (Nov) sniffing stimuli trays containing male breeding season urine.

Behavioral response

Over 44 sessions, the three treatment wolves sniffed the stimuli trays in 70 bouts totaling 158 sec. Wolf #2539 interacted with the trays in 28 bouts totaling 73 sec while her daughters SB#3253 and SB#3254 sniffed in 24 bouts totaling 51 sec and 18 bouts totaling 34 sec, respectively. The number of bouts did not differ by wolf ($\chi^2_2 = 2.17$, $P = 0.34$); however, the total number of seconds spent sniffing was significantly different across the three females ($\chi^2_2 = 14.52$, $P < 0.001$), with SB#2539 spending significantly more time sniffing than expected by chance and SB#3254 spending less. Average daily sniffing duration differed by month with October, when there was male urine added to the stimuli trays, showing higher levels of interaction ($H_2 = 7.37$, $P = 0.02$) (Figure 16).

Average sniffing bout duration showed a similar pattern where bouts were 1.5 times longer in October (treatment) than in September (pre-treatment) and 3.9 times longer than November (post-treatment) ($H_2 = 17.34$, $P < 0.01$).

Wolf #2539 (the dam) was responsible for all instances of urine scent marking (n = 26). She marked most often in November (0.90 ± 0.37 marks/day) as compared with September (0.48 ± 0.19 marks/day) or October (0.46 ± 0.18 marks/day). These differences were not statistically significant ($H_2 = 1.76$, $P = 0.41$), likely due to small sample size. Most often urine scent marks were deposited on the single tree in the enclosure (57.7%), while 26.9% of urine marks were on den structures.

Over the three-month study period, 15 sessions totaling 897 min were coded for state behaviors, proximity to trays, and proximity to other wolves. Wolves were active on average 60.4 ± 3.1% of the time. Wolf #2539 was active more of the time than her daughters, $H_2 = 7.69$, $P = 0.02$ (Figure 17). Wolf #2539 showed a mean proportion of time spent active of 71.0 ± 9.6% compared with SB#3253 (55.2 ± 8.9%) and SB#3254 (54.9 ± 8.8%). For each wolf, proportion of time spent active was consistent across the three months (SB#2539: $H_2 = 1.05$, $P = 0.59$; SB#3253: $H_2 = 0.32$, $P = 0.85$; SB#3254: $H_2 = 0.47$, $P = 0.79$) (Figure 17).

Wolves did not allocate time equally among the six stimuli trays. Overall, the three maned wolves spent more time in proximity to stimuli trays located in the upper yard of the enclosure (Figure 13); 63.7% of the time trays 1 – 3 were the closest tray to the wolf compared with only 36.3% of the time spent in closest proximity to trays 4 – 6. Each individual demonstrated spatial preferences as to where they spent their time ($\chi^2_{10} =$

356.07, $P < 0.01$). Wolf #2539 spent more time than expected by chance closest to tray 6 near where she often slept while SB#3253 spent more time closest to tray 4. Wolf #3254 spent the vast majority of her time (35.8%) in proximity to the tray 3, nearest her den, den 2. Wolves #2539 and #3253 spent less time than expected by chance in proximity to tray 3.

In general, maned wolves spent the vast majority of their time farther than two body lengths away from conspecifics (93.5 ± 0.15%). Wolf #2539 and daughter #3254 spent the least amount of time within two body lengths (3.2 ± 0.79%). The dam and other daughter (SB#3253) spent slightly more time within two body lengths (4.1 ± 0.74%), while the daughters spent 4.8 ± 0.74% of their time in close proximity to one another. However, these differences were not statistically significant ($H_2 = 2.53$, $P = 0.28$). There was also no difference in the proportion of time wolves spent in close proximity to each other across months ($H_2 = 0.28$, $P = 0.87$).

Over 15 sessions, 128 agonistic encounters between the three wolves were recorded and described. Encounters were evenly distributed across months ($\chi^2_2 = 1.00$, $P = 0.61$). Out of all of the encounters, 28.7% involved access through the guillotine gate between the lower and upper yards of the enclosure. In the vast majority of encounters (75.0%) the wolf with the smallest body size, SB#3254, assumed the lower body posture. Of those encounters, 58.6% were between the dam (SB#2539) and SB#3254 and 16.4% were between the sisters. The dam and daughter #3253 had roughly equal interactions where SB#2539 assumed the higher body posture (11.7%) and when SB#3253 was in the higher position (13.3%).

Discussion

Considering the hormonal and behavioral responses together, results suggested some effect of exposure to male urine. Female #2539 showed reproductive hormone profiles indicating some ovarian activity, demonstrated interest in the urine stimuli, and frequently urine scent marked in the month following exposure to the male urine. The lack of clear hormonal and behavioral responses to male urine stimuli in the treatment daughters could be attributed to reproductive suppression by the older, dominant female. This study provided the first evidence of reproductive suppression in co-housed female maned wolves, supported by clear behavioral evidence of social tension. Overall, although daily exposure to 36 mL of this male's urine for 30 days was not sufficient to prompt ovulation in the treatment females, the behavioral interest and intriguing hormonal response of SB#2539 support the hypothesis that urine contains reproductive chemical messages.

There were several factors in the present study that may have confounded the results. These include timing of urine presentation, amount of urine used, age and storage procedures of urine samples, pairing status and relatedness of the male donor, and age and housing status of the females. Clearly more research is needed to tease apart the effects of these confounding variables on reproductive chemical messaging. A systematic look at the impact of co-housing female maned wolves on their reproductive hormones is also strongly advised to test if reproductive suppression happens in this this naturally solitary species.

Lack of ovulation induction in response to urine stimuli

While female SB#2539 showed very intriguing hormone profiles indicating some ovarian activity, overall progestagen concentrations were lower than those reported previously for paired females. The current treatment females showed an average FPM concentration of 0.96 ± 0.10 μg/g feces and controls had 1.15 ± 0.06 μg/g feces. On her own, SB#2539 had average FPM of 1.68 ± 0.25 μg/g feces. Females paired with a male in previous studies showed much higher concentrations, 19.90 ± 1.87 μg/g (Reiter 2012), 20.99 ± 3.60 μg/g (Songsasen et al. 2006), 32.53 ± 2.77 μg/g (Johnson et al. 2014a). For fecal estrogen concentrations, the treatment females showed an average of 58.50 ng/g feces while the only other previous study to report fecal estrogen concentrations with the same antibody showed average concentrations of 274.64 ng/g feces (Johnson et al. 2014a). It should be noted that the females in the Johnson et al. (2014a) study were treated with a GnRH agonist implant. Taken together, it seems that presentation of male urine alone is not enough to simulate the full effect of a male's presence. Defining ovulation as a rise above baseline for at least three consecutive days (Johnson et al. 2014a), none of the females in the present study ovulated in response to the urine stimuli.

In terms of behavioral interest in the scent stimuli trays, the female maned wolves spent more time sniffing (6.15 sec/h) when male urine was present compared to when clean sand was presented alone (pre-exposure: 3.35 sec/h; post-exposure: 1.00 sec/h) suggesting that this stimuli is interesting. A bout of interaction with the stimuli trays began when the subject was within two body lengths of a tray and the "sniff object" behavior was defined as the wolf lowering its head and nose towards the stimulus tray. It is possible that this definition of an interaction was too narrow. Canids, including maned

wolves, have a notoriously strong sense of smell so they likely do not need to closely approach an odor stimulus to adequately access its message. However, previous studies have used even more restrictive definitions of a sniff, within 30 cm (Jordan et al. 2013), so the definition used for the present study still seems reasonable.

Taken together, the hormone concentrations and behavioral interest suggest that the urine stimuli used here did prompt some ovarian activity and sustained behavioral interest but was not sufficient to prompt ovulation. Because SB#2539 showed significantly higher hormone activity in December than in other months, the peak breeding season is suspected to be later than October for these females. This study should thus be repeated later in the season to determine if male urine can prompt ovulation if presented at the height of a female's natural breeding season. The sight of a male alone is not enough to prompt ovulation and contact to scent marks is required for this species (Songsasen, pers. comm.). However, it is possible that there is a visual stimulus (i.e., sight of the male) that must be coupled with the proper chemical cues in order to induce ovulation. In the ewe (*Ovis aries*), visual contact coupled with olfactory access to ram wool prompted ovulation more quickly than olfactory stimulation alone (Pearce and Oldham 1988).

It is also possible that maned wolf urine could be a sufficient ovulation induction stimulus but the urine used for this study may have lacked the critical signaling compounds or may have signaled something that would decrease the likelihood of attraction (i.e., relatedness). The urine used for this study was collected from one male over breeding seasons 2010 and 2011 when he was unpaired (see Appendix 6: Chemical

analysis of urine used in Chapter 4). Urinary chemicals signal dominance status, relatedness, clan membership, and indicate complex social groupings (Sun and Müller-Schwarze 1998; Burgener et al. 2008; Setchell et al. 2010; Theis et al. 2012; Jordan et al. 2013). Thus, it is plausible that this male's urinary chemicals lack the cue to prompt estrus or ovulation in these females due to his relatedness or his single social status.

Additionally, these urine samples were frozen after collection until they were defrosted for use in 2014. Although many urinary volatile organic compounds have been shown to be stable through the freeze-thaw process over a few hours or days (Smith et al. 2008; Alwis et al. 2012), it is possible that the length of time these samples were frozen destroyed the critical chemical signals (Appendix 7: Effects of freezing and ageing on maned wolf urinary VOCs).

The study should be replicated using urine from an unrelated male and the effect of an added visual stimulus should be tested to determine if relatedness or lack of visual stimulus was the reason behind failure to induce ovulation found here. If reproductive suppression does affect female maned wolves, that could explain the lack of ovarian response in the two treatment daughters, and would strongly point to the need for a replication study using only singly-housed females.

If possible, more than 36 mL of urine should be used daily and should be sprayed throughout the enclosure for maximal exposure and to mimic the method of natural deposition. In the present study, females were kept in smaller yards with urine stimuli for approximately 6 h daily but were provided access to a large enclosure with no male urine marks for the other 18 h each day. Thus, it is possible that these females were not

exposed to urine stimuli long enough each day. Future studies comparing behavioral interest in fresh urine collected from unrelated males who were either paired or unpaired with a female would better help to elucidate the reproductive chemical messages contained in the urine of this species.

Evidence of reproductive suppression

Published reports of endocrine and behavioral correlates of group housing do not yet exist for this species, and reproductive suppression has only been studied in cooperatively breeding, social canids. Reproductive suppression is a phenomenon where reproduction of subordinate females is depressed via the presence of the dominant female (Creel and Creel 1991; Creel and MacDonald 1995; Saltzman 2010). This study documents the first investigation into the effects of group housing on reproductive hormones and behavior for the maned wolf. This species is naturally solitary, with juveniles dispersing before age two (Brady and Ditton 1979; Dietz 1984; Bestelmeyer 2000; Emmons 2012). In this case, two daughters remained housed with their mother past the age of natural dispersal and the two pairs of sisters in the control group were maintained together well into their adult years. This is a common practice in zoological institutions where sibling pairs and trios are frequently housed together (Rodden et al. 2007). Though aggression is a known problem for co-housed females (Rodden et al. 2007), this is the first study to quantify agonistic encounters in an attempt to measure social tension.

The FPM levels found here for females housed with other females (treatment: 0.96 ± 0.10, control: 1.15 ± 0.06 μg/g dried feces) are, on average, lower than values

previously reported for singly housed females (3.1 ± 0.4 μg/g in Songsasen et al. 2006 and 3.9 ± 0.35 μg/g in Reiter 2012). The reason for this difference could be attributed to reproductive suppression. In two of the three groups, one female showed higher hormone metabolite levels than the others. In the treatment trio this effect was especially strong; the dam (SB#2539) showed higher concentrations of both FEM and FPM than her daughters. Because the daughters were two years old and showed far lower hormone metabolite levels than those previously published for single females, the presence of the dam could have delayed puberty in her daughters. In one pair of sisters SB#3131 had higher FPM than her sister (but lower FEM), however, in the other sister pair there were no differences in either hormone. Housing sisters together could suppress reproduction in one or both of the siblings, potentially exacerbated by lack of exposure to a male and lack of reproductive experience.

Behavioral evidence from the treatment trio also indicates reproductive suppression. The mother wolf (SB#2539) was more active than her daughters, interacted more with urine stimuli, adopted a higher body posture in most conspecific interactions, and was responsible for depositing all of the urine scent marks during the study. These findings suggest that the dam was the dominant individual in the group, patrolling and scent-marking her territory. Although the daughters were adults during this study (two years old), they likely remain subordinate to their mother while living in the same enclosure. These results are consistent with previous canid research suggesting that dominant individuals scent mark most frequently (Asa et al. 1984b; Asa et al. 1990; Gese and Ruff 1997; Parker 2010; Jackson et al. 2012; Jordan et al. 2013), scent marking

frequency is related to levels of reproductive hormones (Asa et al. 1990), and only maned wolf territory holders scent-mark (Bestelmeyer 2000).

This study provided the first evidence that reproductive suppression can occur when individuals of a solitary canid species are housed together. However, because one pair did not exhibit any hormonal differences and behavioral observations were only conducted for one of three groups, the evidence in favor of reproductive suppression is not definitive. Hormone concentrations for all the females were far below those of females paired with males. The traditional definition of reproductive suppression where one dominant female cycles while others are reproductively suppressed does not apply to induced ovulating species like the maned wolf. These findings underscore the need for a more comprehensive study investigating the effects of group housing on behavior and hormones. In this proposed study, in addition to estrogen and progestagen metabolites, cortisol should also be measured to further investigate the hypothesis of social stress for co-housed female maned wolves.

The treatment individuals practiced active avoidance, spending the vast majority of their time farther than two body lengths away from conspecifics and avoiding areas preferred by one of the trio. Wolves #2539 and #3253 spent less time than expected by chance in proximity to the den preferred by wolf #3254 and she spent much more time in this location. Each individual showed strong spatial preferences, corroborating findings by the Maned Wolf Species Survival Plan that access to multiple dens is beneficial to the species (Rodden et al. 2007). There were many instances where SB#3254 escaped an agonistic encounter by entering her den, thereby de-escalating the conflict. There were on

average 8.5 agonistic encounters per hour and around 29% of those involved the gate separating the upper and lower yards of the enclosure. A second recommendation arising from this work is to facilitate access between yards and buildings by installing or opening multiple gates. Installing multiple access points between locations should enable co-housed individuals to continue to avoid each other and decrease conflict. It should be noted that during the behavioral observations the trio was confined to a smaller section of their total enclosure to maximize the probability that they would interact with the scent stimuli. It is not known what the rate of agonistic encounters would be if wolves were provided their usual access to the larger enclosure.

The findings of this study open up multiple avenues for future research. The male urine used here was not sufficient to prompt ovulation, but ovarian activity was seen for the dominant treatment female, and behavioral interest in the odor stimuli was recorded for all three treatment females. Future studies should compare female responses to urine from unrelated males who were paired with a female to those who were unpaired to determine if there is a pairing prerequisite to producing an ovulation induction signal. Mammalian urine is known to contain chemical signals indicating social dominance (Setchell et al. 2010; Apps et al. 2012; Jordan et al. 2013) and reproductive status (Schultz et al. 1988; Bagley et al. 2006; Crawford and Drea 2015), so it is entirely plausible that there are urinary compounds that signal paired status.

Fresh male urine could also be used in case critical signaling elements are mediated by bacteria as in the hyena (*Crocuta crocuta*) (Theis et al. 2013), in which case

the freezing process would disrupt the signals. Further studies should determine the necessity of a visual stimulus coupled with olfactory signals.

A more comprehensive study with increased sample size of family trios and sister pairs should be conducted to ascertain the extent of reproductive suppression in this species. If reproductive suppression affects group-housed females, this study would need to be repeated with all adult females each housed singly. Each of these additional studies would provide clues about the mechanism behind this fascinating induced ovulation adaptation in this unique species.

CHAPTER 5: CONCLUSIONS

This dissertation is comprised of three major studies connected by the theme of investigating chemical communication about reproduction in the maned wolf (*Chrysocyon brachyurus*). There were three main objectives: (1) Identify volatile organic compounds (VOCs) that are consistently found in maned wolf urine; (2) Examine differences in VOCs according to sex and reproductive status to distinguish putative semiochemicals; and (3) Investigate the behavioral and hormonal responses of female maned wolves to male urine stimuli. Chapter 2 examined the volatile chemical constituents of monthly urine samples from 11 maned wolves to assess the typical composition of maned wolf urinary VOCs and investigate the differences between male and female urinary VOCs. Chapter 3 expanded on the findings of the first study through the analysis of weekly samples from 13 maned wolves and updated chemometric analyses. Finally, Chapter 4 assessed the hormonal and behavioral responses of female maned wolves to exposure to male urine and co-housing females.

Collectively this body of work provides the most comprehensive analysis of maned wolf urinary VOCs to date and identifies several putative semiochemicals that may indicate sex, male reproductive status (intact versus castrated), or pairing status. Chapters 2 and 3 are the first investigations of the biological functions of maned wolf urinary VOCs. Behavioral and hormonal evidence from Chapter 4 supports the

hypothesis of the presence of urinary chemical signals in male maned wolf urine that play significant roles in estrus or ovulation induction. Additionally, Chapter 4 provides the first evidence of reproductive suppression in co-housed female maned wolves.

Ubiquitous urinary VOCs

Chapter 2 analyzed the VOCs found commonly in the maned wolf. Around half of the compounds were pyrazines, heterocyclic nitrogen-containing compounds usually with a strong odor. Pyrazines are common flavor additives in the food industry (Maga et al. 1973; Liu et al. 2011) and are quite common as chemical signaling compounds in insects as well as mammals (Guilford et al. 1987; Brophy 1989; Yamada et al. 1989; Woolfson and Rothschild 1990; Osada et al. 2013). Along with pyrazines, hemiterpenoid alcohols and ketones were common. Many of these compounds had been previously identified in maned wolf urine (Childs-Sanford 2005; Goodwin et al. 2013). Although many compounds had not previously been reported in maned wolf urine, some are commonly found in other mammalian secretions. For example, 4-heptanone and 6-methyl-5-hepten-2-one are constituents of urine from a wide variety of carnivores (Jorgensen et al. 1978; Raymer et al. 1984; Zhang et al. 2005; Burger et al. 2006; Parker 2010) and acetophenone is common in urine or feces from other canids (Raymer et al. 1984; Schultz et al. 1988; Parker 2010; Martín et al. 2010). It is not yet known what evolutionary significance may be attributed to the presence of urinary VOCs that are found in some species but not others.

The compounds common to all maned wolves in Chapter 2 may confer non-reproductive signals among conspecifics. Because many of these compounds are the most

abundant in maned wolf urine and are aromatic, this suite of compounds is likely responsible for the characteristic odor of maned wolf urine. These compounds could serve as a suitable control scent or background signal for future behavioral bioassays.

Differentially expressed urinary VOCs

This dissertation adds valuable information to the growing body of knowledge of mammalian semiochemistry. The maned wolf is only the fifth species (out of 36) within the Canidae to be investigated for urinary VOCs. Few of the existing canid studies have attempted to analyze differential expression by sex (excepting Raymer et al. 1984; Raymer et al. 1986; Parker 2010; Apps et al. 2012), and none have done so in the maned wolf.

Chapters 2 and 3 both assessed differences in maned wolf VOCs by sex. Chapter 2 was based on 103 samples from 11 maned wolves while Chapter 3 included 332 samples from 13 maned wolves. Compared to typical mammalian chemical ecology studies, usually based on tens of samples, these datasets represent some of the most robust sampling in this field. Because each sample was run in triplicate the resulting datasets were quite large. In the analyses of Chapter 2, a large batch effect was observed where samples run on the same date were more similar to each other than to samples from the same individual wolf run on different dates. Retention time correction and normalization procedures helped correct for these effects and this problem was fully resolved for the analyses of Chapter 3. These large datasets required data processing and analysis steps commonly used in disease metabolomics studies, but not yet mainstream for mammalian signaling studies. Therefore, the data analysis pipeline presented in

Chapter 3 provides an excellent guide for mammalian semiochemical researchers to use to improve their ability to derive meaning from complex, noisy datasets. Appendix 3 re-analyzed a subset of data from Chapter 2 using the improved data analysis pipeline developed in Chapter 3.

In all three analyses, 2-ethenyl-6-methyl pyrazine and 2-nonen-4-one were higher in abundance in males than females. 2-Ethenyl-6-methyl pyrazine is one of the most abundant VOCs in maned wolf urine (Goodwin et al. 2013). 2-Ethenyl-6-methyl pyrazine has been previously identified in both mammals and insects. It is present in the urine of sexually intact male brown antechinus (*Antechinus stuartii*, family Dasyuridae) and was not identified in female conspecifics (Toftegaards et al. 1999). 2-Ethenyl-6-methyl pyrazine is also a reproductive semiochemical in the papaya fruit fly (*Toxotrypana curvicauda*) (Robledo and Arzuffi 2012). 2-Nonen-4-one is well known as a volatile constituent of fruits and vegetables (Buttery et al. 1970; Buttery et al. 1971). It is also a putative reproductive semiochemical found in the rutting pits of male Alaskan moose (*Alces alces gigas*) (Whittle et al. 2000) and is also found in the urine of some European badgers (*Meles meles*) (Service et al. 2001). In the analyses of both Chapters 2 and 3, 2-methyl-3-buten-2-ol was higher in relative abundance in males than females. 2-Methyl-3-buten-2-ol is a constituent of the interdigital gland secretion of both male and female red hartebeest (*Alcelaphus buselaphus*) (Reiter et al. 2003) and is a pheromone in several hundred species of insect (e.g. Birgersson et al. 1984; Pajares et al. 2010). The evolutionary purpose behind the finding that certain urinary VOCs are present in some species but not others has not yet been elucidated. The development of a single database

where researchers can deposit information about semiochemical presence in each species is underway (www.pherobase.com).

4-Heptanone had higher abundance in females in all three analyses. This compound has been previously identified in urine across a wide variety of carnivores. Differences in the abundance of 4-heptanone between the sexes are known for some species. In the cheetah (*Acinonyx jubatus*) this compound is only found in female urine (Burger et al. 2006), while in the ferret (*Mustela furo*) 4-heptanone is more abundant in males than in females (Zhang et al. 2005). In the red fox (*Vulpes vulpes*), 4-heptanone is identified in both males and females but there is a difference in abundance for males between breeding and non-breeding seasons (Jorgensen et al. 1978).

10-Methyl-2-oxecanone and 3-iodo-E-2-octenoic acid showed the same male bias in Appendix 3 as was found in Chapter 3. These two VOCs were not identified in the original Chapter 2 analysis. While no literature references could be found for 3-iodo-E-2-octenoic acid, it is important to remember that the identifications presented here are based on spectral library searches and still need to be verified by analytical standard wherever available. 10-Methyl-2-oxecanone is the main component of the femoral gland of the Madagascan frog (*Mantidactylus femoralis*) (Poth et al. 2013), is a constituent of the odiferous defensive secretion of the eucalypt longicorn beetle, *Phoracantha synonyma* (Kitahara et al. 1983), and is produced by green mold (*Trichoderma spp*.) during periods of mycelia growth (Radványi et al. 2015). To our knowledge, this compound has not yet been reported in mammalian secretions.

The three analyses were not expected to exactly replicate each other because between Chapters 2 and 3 the datasets were different and the samples were analyzed on different GC columns. Nonetheless, many of the same VOCs were found across these three analyses and there were no VOCs that contradicted findings of previous chapters. Taken together, these results provide confidence that the urinary VOCs of maned wolves differ significantly based on sex, with males producing higher abundances of many VOCs as compared with the females.

Striking differences were noted between intact males and a castrated male; specifically the castrated male produced lower abundances of many VOCs, suggesting that these compounds may be dependent on testosterone. Further, the castrated male lacked several VOCs that are common in maned wolf urine. For example, 2-methyl-6-(1-propenyl) pyrazine is one of the main volatile components of maned wolf urine (Childs-Sanford 2005; Goodwin et al. 2013) and was 124-fold less abundant in the urine of castrated males compared to intact males. Even to the human nose, differences between the castrated male urine samples and those from intact males were easily detected; the urine from the castrated male did not smell strongly at all while the urine sample from intact males were quite pungent.

Future studies should correlate these sex-biased VOCs to reproductive hormone levels and should test these compounds in behavioral bioassays to ascertain their dependence on steroid hormones and their relevance to reproduction in the maned wolf.

Chemosignals that may induce estrus or ovulation

This dissertation was limited in its ability to detect VOCs that may play a role in estrus or ovulation induction in the maned wolf. Sample sets in Chapters 2 and 3 only had one pair that successfully bred each year and it was the same pair in both years (male SB#2660 and female SB#2945). Therefore, it is possible that breeding specific compounds were confounded with individual-specific compounds. Building on this work, future analytical chemistry studies should strive to collect samples from several breeding pairs with increased frequency around the time of breeding to better ascertain which VOCs may play a role in maned wolf reproduction.

Despite this limitation, Chapter 3 provides a foundation for selecting VOCs that may play a role in estrus or ovulation induction in the maned wolf. 1,1'-thiobis-cyclopentane and 1-(2-hydroxy-5-methylphenyl)-2-buten-1-one are the strongest candidates for bioassay testing. 1,1'-Thiobis-cyclopentane had an 8-fold higher relative abundance in male maned wolves compared to females and over a 200-fold increase in relative abundance in intact males compared to the castrated male. Further, this compound peaked on the day of breeding for both individuals that successfully bred during 2014. Behavioral bioassays should show significant behavioral interest in these compounds and if they are responsible for estrus or ovulation induction, endocrine profiles of singly housed females should demonstrate ovarian activity indicative of estrus (sharp peak in estrogen levels) or ovulation (prolonged elevation of progestagens).

In Chapter 4, three co-housed females were exposed to male urine daily. The hormonal and behavioral responses of the females suggest an effect of exposure to male urine on ovarian activity and behavior. Specifically, the dam in the treatment group

showed reproductive hormone profiles indicating some ovarian activity, demonstrated interest in the urine stimuli, and frequently urine scent marked in the month following exposure to the male urine. Although daily exposure to 36 mL of male urine for 30 days was not sufficient to prompt ovulation in any of the treatment females, the behavioral interest of all three treatment females and the intriguing hormonal response of the dam support the hypothesis that male urine contains reproductive chemical messages.

The findings of this study open up multiple avenues for future research. It is possible that the relatedness of the male urine donor affected female responses. Additionally, perhaps females were not exposed to sufficient urine stimuli each day, so in future studies, more than 36 mL of urine should be used daily and should be sprayed throughout the enclosure for maximal exposure and to mimic the method of natural deposition. Further studies are also needed to determine if there is a visual stimulus (i.e., sight of the male) that must be coupled with urinary chemical cues in order to induce ovulation. Together, these studies would help to elucidate the reproductive chemical messages contained in the urine of this species.

Reproductive Suppression

Chapter 4 provides the first potential evidence of reproductive suppression in co-housed female maned wolves, supported by clear behavioral evidence of social tension. Published reports of endocrine and behavioral correlates of group housing do not yet exist for this species, and reproductive suppression has only been studied in cooperatively breeding, social canids. The maned wolf is naturally solitary (Brady and Ditton 1979;

Dietz 1984; Bestelmeyer 2000; Emmons 2012), but is commonly housed in pairs or trios in zoological institutions (Rodden et al. 2007).

The fecal progestagen levels found here for co-housed females were lower than values previously reported for singly housed females (Songsasen et al. 2006; Reiter 2012). Fecal estrogen concentrations using an estrone conjugate antibody have not been reported for single females. This difference could be attributed to reproductive suppression. In the treatment trio the dam showed much higher concentrations of both estrogens and progestagens than her daughters, suggesting that perhaps the presence of the dam delayed puberty in her daughters. Housing sisters together could suppress reproduction in one or both of the siblings. In one pair of sisters, one individual had higher progestagens than her sister; however, in the other sister pair there were no differences in either hormone. Because one sister pair did not exhibit any hormonal differences and behavioral observations were only conducted for the trio, the evidence in favor of reproductive suppression is not definitive. These findings underscore the need for a more comprehensive study investigating the effects of group housing on behavior and hormones.

Behavioral evidence from the treatment trio also indicates reproductive suppression. Though aggression is a known problem for co-housed maned wolves (Rodden et al. 2007), this is the first study to quantify agonistic encounters in an attempt to measure social tension in group-housed females. In the treatment trio, the mother wolf was more active than her daughters, interacted more with the male urine stimuli, adopted a higher body posture in most conspecific interactions, and was responsible for

depositing all of the urine scent marks during the study. These findings suggest that the dam was the dominant individual in the group, patrolling and scent-marking her territory. Although the daughters were adults during this study (two years old), they likely remained subordinate to their mother while living in the same enclosure.

The trio practiced active avoidance, spending the vast majority of their time farther than two body lengths away from conspecifics and avoiding areas preferred by one individual. These findings corroborate the recommendation by the MWSSP that access to multiple dens is beneficial to the species (Rodden et al. 2007). There were on average 8.5 agonistic encounters per hour and many of those involved access to a gate separating the yards of the enclosure. A second recommendation arising from this work is to facilitate access between yards and buildings by installing or opening multiple gates. Multiple access points between locations would enable co-housed individuals to continue to avoid each other and decrease conflict.

A systematic study with increased sample size of family trios and sister pairs should be conducted to ascertain the extent of reproductive suppression in this species. In addition to behavior and reproductive hormones, cortisol should also be measured to further investigate the hypothesis of social stress for co-housed female maned wolves. If reproductive suppression affects group-housed females, studies of the effects of exposure to male urine on ovarian function and behavior would need to be repeated with adult females each housed singly. These additional studies would provide clues about the mechanism behind this fascinating induced ovulation adaptation in this unique species.

Context

The solitary lifestyle of the maned wolf means that induced ovulation would be adaptive. Induced ovulation is a relatively rare feature within Mammalia (Conaway 1971; Larivière and Ferguson 2003). Although there are species exhibiting induced ovulation throughout several families within Mammalia, most are thought to have a copulatory mechanism. For example, this is the case for many carnivores in Felidae (Bakker and Baum 2000; Brown 2011) and Mustelidae (Amstislavsky and Ternovskaya 2000; Larivière and Ferguson 2003) as well as for several rodent species in Bathyergidae (Malherbe et al. 2004; Jackson and Bennett 2005; van Sandwyk and Bennett 2005) and lagomorphs (Conaway 1971; Sawyer and Radford 1978; Ramirez and Soufi 1994; Melo and González-Mariscal 2010). Many induced ovulating species in Camelidae ovulate in response to a protein in seminal fluid, now termed the ovulation induction factor (OIF) (Pan et al. 2001; Larivière and Ferguson 2003; Senger 2003; Adams et al. 2005; Nagy et al. 2005; Adams and Ratto 2013). There are only two induced ovulating species known to have a pheromonal cue to prompt ovulation: the gray short-tailed opossum (*Monodelphis domesticus*) (Stonerook and Harder 1992; Jackson and Harder 1996; Harder and Jackson 2003; Harder et al. 2008; Vitazka et al. 2009) and the prairie vole (*Microtus ochrogastor*) (Richmond and Conaway 1969), though an olfactory mechanism has been hypothesized for the dromedary camel (*Camelus dromedarius*) (Adams and Ratto 2013).

In many spontaneously ovulating mammals, male chemosignals affect estrus and ovulation. This is the case for several rodent species: mice (*Mus musculus*) (Marsden and Bronson 1964; Marsden and Bronson 1964; Bronson and Whitten 1968; Whitten et al. 1968; Jemiolo et al. 1985; Jemiolo et al. 1986; Marchlewska-Koj et al. 1990; Ma et al.

1999; Marchlewska-Koj et al. 2000; Morè 2006), deermice (*Peromyscus maniculatus*) (Bronson and Marsden 1964), rats (*Rattus norvegicus*) (Johns et al. 1978), voles (*Microtus* spp.) (Carter et al. 1980; Lepri and Vandenbergh 1986; Lepri and Wysocki 1987; Lyons and Getz 1993), and Siberian hamsters (*Phodopus sungorus*) (Dodge et al. 2002), as well as for sheep (*Ovis aries*) (Cohen-Tannoudji et al. 1989; Cohen-Tannoudji et al. 1994; Gelez et al. 2004; Gelez and Fabre-Nys 2006), and goats (*Capra aegagrus hircus*) (Iwata et al. 2000; Murata et al. 2009; Bedos et al. 2010; Murata et al. 2014).

No carnivores studied to date have an olfactory mechanism underlying estrus or ovulation. Therefore, if the maned wolf does indeed have an olfactory mechanism to induce estrus or ovulation, this would represent a significant addition to the fascinating list of reproductive mechanisms and adaptations in canids and carnivores. The body of knowledge in this dissertation does support the hypothesis of an olfactory mechanism prompting estrus or ovulation in the maned wolf. This finding has implications for the evolution of this unique trait within the family Canidae, and across Carnivora and Mammalia.

APPENDIX 1: OPTIMIZATION OF HEADSPACE SOLID-PHASE MICROEXTRACTION FOR MANED WOLF URINARY VOC ANALYSIS

Introduction

Optimizing the analysis of volatile organic compounds (VOCs) from the sample headspace (gas above liquid urine) involves a number of choices to be made regarding the exact methods needed. The choice of coating on the solid phase microextraction (SPME) fiber and the temperature during VOC extraction from the headspace are important considerations in an analysis.

In a headspace SPME analysis, the urine sample is heated to a given temperature and held there so that the VOCs reach equilibrium between the liquid urine and the headspace above the urine. Then the temperature is held constant while the SPME fiber is exposed to the headspace so that the VOCs attain equilibrium between the headspace and the SPME fiber coating. Two extraction temperatures were tested, the internal body temperature of a wolf, 37°C (Goodwin et al. 2005), and a higher temperature, 60°C (Dixon et al. 2011).

Several SPME fiber coatings are commercially available, including polyacrylate (PA), polydimethylsiloxane (PDMS), polyethylene glycol (PEG), and mixed phases of carboxen (CAR)/PDMS, divinylbenzene (DVB)/PDMS, and CAR/DVB/PDMS. Each fiber coating has a different affinity for different analytes depending in large part on the polarity of the analyte (Alpendurada 2000). Therefore, the choice of the fiber coating will

dictate which types of chemical classes are adsorbed onto the fiber to be injected into the GC-MS.

Because metabolomic analyses attempt to isolate and identify the widest array of chemical constituents from a variety of chemical classes, choosing the optimal extraction temperature and a SPME fiber coating that maximizes the number of analytes identified is desirable.

Extraction temperature test

Methods

Urine from one female maned wolf, SB#2845, and one male wolf, SB#2814, collected during 2010 were used. These samples had been frozen at -20°C from collection until processing and running in April 2013. Urine samples were divided into 4 aliquots of 2 mL each. Aliquots from each wolf were run in duplicate at the two temperatures and a water blank was run for each sample-temperature combination. Prior to headspace analysis the 50/30 μm CAR/DVB/PDMS SPME fiber was conditioned according to manufacturer recommendations (Table 13). This way, the SPME fiber was clean from any VOCs prior to the experiment.

Prior to use, glass headspace vials were rinsed with ultrapure water, rinsed with methanol, and baked at 425°C overnight. Each urine replicate was heated for 30 min and then SPME extraction lasted 45 min. This extraction time was shown to have the best combination of the number of compounds identified in the GC-MS run balanced by the efficiency of sample throughput (Dixon et al. 2011). Samples were run on an RTX-VMS GC column (Restek Corporation, Bellefonte, PA) using the same methods as in Chapter

2. Chromatographic deconvolution and compound identification were carried out for each replicate using the Automated Mass Spectral Deconvolution and Identification System (AMDIS ver 2.69) software and the mass spectral library (NIST11).

Table 13. Conditioning procedures for SPME fiber coatings recommended by Restek Corporation (Bellefonte, PA).

Thickness (µm)	Coating	Precondition Temp (°C)	Precondition Time (h)
75	CAR/PDMS	300	1
85	CAR/PDMS	300	1
7	PDMS	320	1
100	PDMS	250	0.5
65	PDMS/DVB	250	0.5
85	PA	280	1
50/30	DVB/CAR/PDMS	270	1
60	PEG	240	0.5

Results and Discussion

Following the data analysis protocol detailed in Chapter 2, the resulting dataset contained 177 compounds. There was a significant effect of temperature $t_{2825} = -2.27$, $P = 0.023$ where 60°C had slightly higher mean normalized intensity values of the VOCs, meaning that the abundances of the VOCs were higher on average for this temperature. There were 6 compounds that were only identified at 37°C while 19 compounds were only identified at 60°C.

Importantly, however, we noticed a severe bulging of the septa at the 60°C temperature that was not apparent at the lower temperature. Due to the increased pressure inside the vials, when septa were pierced with the SPME fibers, liquid urine would often splash in the vial and would contact the fiber. Because our aim was to measure the

headspace volatiles, liquid urine on the SPME fiber was not deemed acceptable. Accordingly, headspace VOCs were extracted at 37°C for the following studies.

SPME fiber coating test

Methods

Urine from male SB#2810 collected on 11/21/2011 was pipetted into 2 mL aliquots in cleaned glass headspace vials. This sample had been frozen at -20°C from collection until processing and running in December 2013. Aliquots were run in triplicate with each fiber coating and a water blank was run for each fiber coating. Prior to each run the SPME fiber was conditioned according to manufacturer recommendations (Table 13). Each urine replicate was heated for 30 min followed by SPME extraction for 45 min. This extraction time was shown to have the best combination of the number of compounds identified in the GC-MS run balanced by the efficiency of sample throughput (Dixon et al. 2011). Samples were run on an RTX-VMS GC column (Restek Corporation, Bellefonte, PA) using the same methods as in Chapter 2. Chromatographic peaks were selected for each of the replicate data files via the {xcms} package in R (Tautenhahn et al. 2012). A one-way Analysis of Variance (ANOVA) was conducted to test for significant differences in the number of compounds detected using each of the fiber coatings. The number of peaks identified by the peak-picking algorithm in {xcms} was reported as mean ± standard error of the mean (SEM).

Results and discussion

In total, 24 data files were analyzed, three replicates for each of eight fiber coatings. The 50/30 μm DVB/CAR/PDMS coating enabled identification of the highest number of peaks (918.33 ± 17.46) (Figure 18). This fiber coating identified significantly

more compounds than all of the other coatings except the 65 µm PDMS/DVB fiber which identified close to that of the best fiber (889 ± 19.66) ($F_{7,16}$ = 69.30, P < 0.0001). Based on these data, the 50/30 µm DVB/CAR/PDMS fiber coating was selected for SPME analyses of maned wolf urinary VOCs.

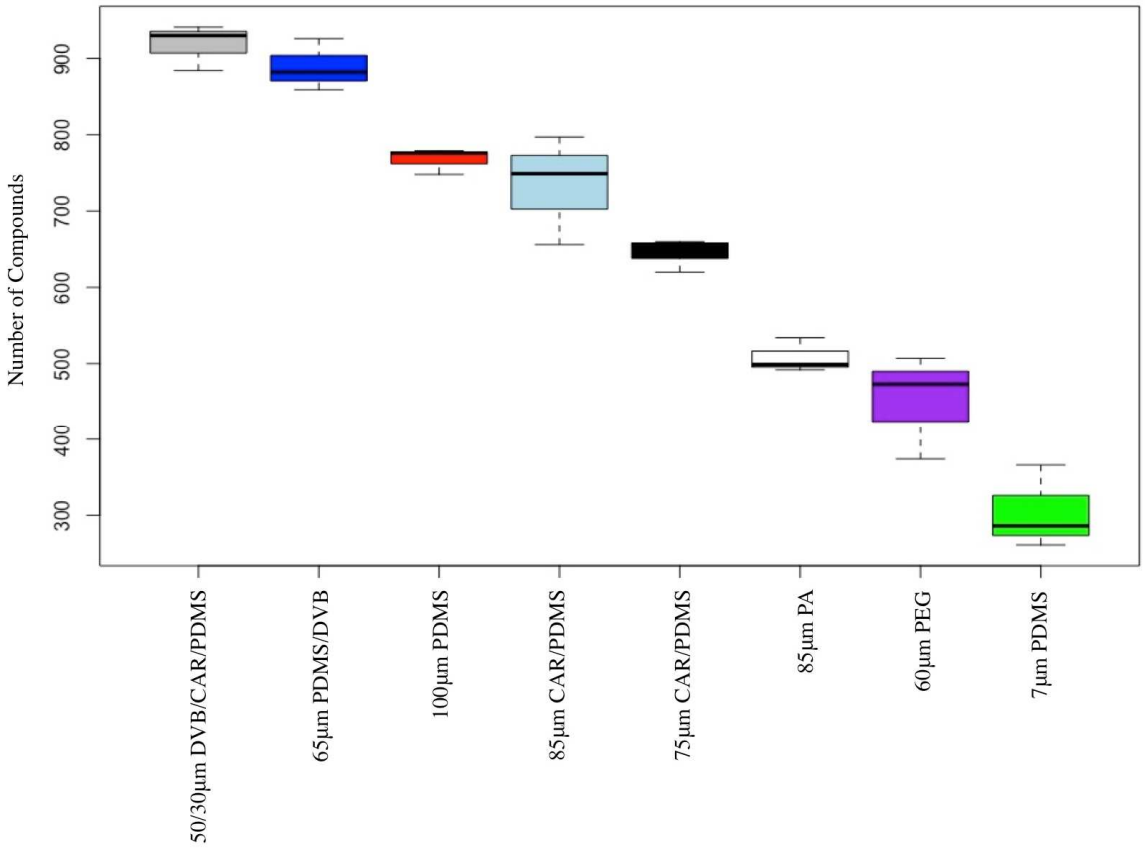


Figure 18. Number of chromatographic peaks identified by {xcms} peak picking algorithm for eight SPME fiber coatings.

APPENDIX 2: DATA ANALYSIS CODE FOR CHAPTER 3

This appendix provides a step-by-step guide to conducting a metabolomics data analysis using open source tools freely available online. Portions of code that must be altered are in red text.

I found that running the peak picking and grouping steps for each sample separately worked best to preserve differences between samples.

STEP 1: XCMS script

Run the following script in R (vers. 3.1.2) for the replicate files from each sample.

```
#############################################################################
#load necessary libraries
library(xcms)
library(metaMS)
library(metaMSdata)

#set path to directory with your replicate files
mzXMLpath <- "path_to_replicate_files_from_1_sample"
mzXMLfiles <- list.files(mzXMLpath, recursive = TRUE, full.names = TRUE)
#create file object
mzXMLfiles #check file object

#############################################################################
#find peaks
xset <- xcmsSet(mzXMLfiles, fwhm = 3, snthresh = 10, max = 100,
       step=0.25, steps=2, mzdiff=0.5)

#check number of peaks per sample
for (i in 1:length(filepaths(xset))) {
  cat(basename(filepaths(xset)[i]),
            length(which(peaks(xset)[,"sample"] == i)), "\n")}

#group peaks so that peaks representing the same analyte across samples are
placed into groups
xset <- group(xset, bw=3)
```

```
#############################################################################
#retention time correction using Obiwarp algorithm
xsetcor <- retcor.obiwarp(xset, profStep = 1, plottype = "deviation")

#############################################################################
#regroup peaks using corrected RTs
xsetcor <- group(xsetcor, bw = 3, mzwid=0.25, minfrac=0.2, minsamp=2)

#############################################################################
#fill in missing peaks
#this function looks at certain RTs where we expect a peak to be and
integrates under the curve at that corrected RT
xsetfill <- fillPeaks(xsetcor)

#############################################################################
#use CAMERA package to deconvolute spectra and assemble ions into compounds
library(CAMERA)

#this function creates a variable pcgroup that tells you which ions are
believed to belong to the same compound
xs <- xsAnnotate(xsetfill)

#group by RT value of the xcms grouped peak
xsaF <- groupFWHM(xs, perfwhm=0.6)

#Verify grouping
xsaC <- groupCorr(xsaF)

#save results into object
rt <- getPeaklist(xsaC)
#############################################################################
#End script
#############################################################################
```

STEP 2: Keep ion with highest abundance (quantitative ion)

Because there are multiple ion fragments that compose each compound, we need to deal with that prior to statistical analysis. So that the resulting file has only one entry per chemical constituent, I decided to retain the ion with the highest abundance and discard the entries for the other ion fragments within each compound. Thus, the abundance of the quantitative ion can be used as a proxy for the abundance of the compound overall in downstream data analyses. Secondly, in this step I removed

"compounds" that were not composed of at least three ions because those are unlikely to represent real chemical compounds.

```
###############################################################################
rt[,1] <- NULL   #delete first row of rt dataframe (entity numbers)

cols = regexpr("_", colnames(rt)) >= 0 #select the columns with replicate data
(all of mine have "_" in the column heading. You may need to change this
depending on how you named your replicates)

rowsums <- rowSums(rt[, cols ]) #sum ions across replicates

rt <- cbind(rt, rowsums)
colnames(rt)[ncol(rt)] <- "rowsums"

###############################################################################
#select row within pcgroup that has the max abundance (quant ion)
max_sel <- ave(rt$rowsums, rt$pcgroup, FUN=max) == rt$rowsums

###############################################################################
#select pcgroups where there are 3 or more ions
ion_sel <- ave(rt$rowsums, rt$pcgroup, FUN=rank) >= 3

###############################################################################
#create resulting dataframe
new_df <- rt[ max_sel & ion_sel ,]

#write the results file
write.csv(new_df, file="path_to_file/result.csv")
###############################################################################
#End script
###############################################################################
```

STEP 3: Average ion abundances over replicates

Using the.csv file resulting from the R script above, I averaged the ion abundances from all replicates of a given sample to provide one measure of average ion abundances for that sample. This process was completed for each sample.

```
###############################################################################
#Average abundance of quant ion in all three reps together for each sample
run_names <- lapply(colnames(new_df)[column numbers containing ion abundances
of replicates], as.character) #make vector composed of all of the names of the
replicates

sample_names <- unique(sub("_[rR][eE][pP][123]$","", run_names)) #remove _rep
number and return unique sample names
```

```
#depending on how your run names are written you may need to change the
arguments to sub. Mine were named as follows samplename_rep1 etc.

###############################################################################
#define an averaging function that returns a column
avgfn <- function(df, sample){
  rowMeans(df[, grep(sample, names(df))] ) #find the columns in the df where
sample names are names of the dataframe
}

data2 <- newdata[,column numbers for columns that do not contain ion
abundances] #don't average these columns because they contain important data
like mz, RT, etc.

dat2 = matrix( nrow = nrow(data2), ncol = length(sample_names))
new_rt <- cbind(data2, dat2)
head(new_rt)

for(i in c(column numbers containing ion abundances of replicates)){
  new_rt[i] <- avgfn(newdata, sample_names[i-last column number in data2])
}

###############################################################################
#  now put the column_names back on
colnames(new_rt) <- c( colnames(new_rt)[1:last column number in data2],
sample_names)

#check that there is now just one entry for each sample
head(new_rt)

#write results file
write.csv(new_rt,file="path_for_results/new_rt.csv")
###############################################################################
#End script
###############################################################################
```

APPENDIX 3: ANALYSIS OF CHAPTER 2 DATA WITH DATA ANALYSIS WORKFLOW FROM CHAPTER 3

Introduction

Because the data analysis pipeline was improved between publishing Chapter 2 and analyzing the data from Chapter 3, an analysis of the Chapter 2 data using the data analysis pipeline from Chapter 3 was conducted.

Methods

Data files from Chapter 2 were run through the {xcms} and {camera} R packages but due to a glitch in the retention time correction step, only 46 samples out of the 103 total samples were processed correctly. These 46 samples (n = 27 male, 19 female) were used in the data analysis steps detailed in Chapter 3. The data analysis methodology was the same as in Chapter 3 except that no internal standard normalization was conducted and the significance parameters ($P < 0.001$ and Fold Change (FC) >3.0) were relaxed to $P < 0.05$ and FC > 3.0. The reason for this change was that the retention times in the Chapter 2 dataset were far more variable than those in Chapter 3, causing a large batch effect in this dataset that obscured the differential expression of VOCs by sex.

To compare the results from the analysis methods used in Chapter 2 to those used for Chapter 3, Chapter 2 samples were analyzed for VOCs that differed between males and females. Thus, results from this analysis should be similar to those in Table 4 and Table 7.

Results and Discussion

Overall, only 28 VOCs were aligned across all the samples due to the batch effect. Of those, eight VOCs differed significantly ($P < 0.05$ and FC >3.0) between males and females (Table 14). Results were quite similar to those in Chapter 2 (Table 4) and Chapter 3 (Table 7). Specifically, 2-ethenyl-6-methyl pyrazine and 2-nonen-4-one were higher in abundance in males than females in the present analysis as well as the analyses in Chapter 2 and Chapter 3. 4-Heptanone had higher abundance in females in all three analyses. 10-Methyl-2-oxecanone and 3-iodo-E-2-octenoic acid showed the same male bias here as was found in Chapter 3. These two VOCs were not identified in the original Chapter 2 analysis. Two VOCs, tetrahydro-2-isopentyl-5-propyl furan and the butyrolactone showed higher abundance in males as compared to females in this analysis and were shown in Chapter 3 to have higher abundance in intact males compared to castrated males. Two additional VOCs that differed significantly between males and females in Chapters 2 and 3 were also found through this analysis, but abundances were not statistically different enough between males and females to be significant. These included 3-ethyl-2,5-dimethyl pyrazine (more abundant in females) and 2-methyl-3-buten-2-ol (more abundant in males).

Table 14. VOCs that differed significantly ($P < 0.05$ and fold change >3.0) between male (n = 27 samples) and female (n = 19 samples) maned wolf urine samples.

Compound[a]	RT	CAS No.	Log2(relative abundance) (mean ± SD)		Fold Change		*P*	VIP on PLS Latent Variable 1	RF Mean decrease in accuracy
			Male (N = 27)	Female (N = 19)	Raw[b]	Log_2[c]			
2-ethenyl-6-methyl pyrazine	14.60	13925-09-2	0.58 ± 0.34	-0.83 ± 0.18	21.90	-4.45	8.27E-04	1.393	0.026
tetrahydro-2-isopentyl-5-propyl furan	25.47	33933-71-0	-0.68 ± 0.36	0.97 ± 0.48	10.38	3.38	9.03E-03	1.630	0.038
±-β,β-dimethyl-γ-(hydroxy-methyl)-γ-butyrolactone	21.75	52398-48-8	-0.67 ± 0.34	0.96 ± 0.44	8.27	3.05	5.62E-03	1.608	0.038
10-methyl-2-oxecanone	30.18	65372-24-6	0.72 ± 0.22	-0.09 ± 0.43	7.67	-2.94	4.01E-06	1.721	0.079
3-methyl butanoic acid	11.70	503-74-2	-0.67 ± 0.27	0.95 ± 0.34	7.37	2.88	5.88E-04	1.602	0.009
3-iodo-E-2-octenoic acid	28.79	NIST ID: 308875	0.43 ± 0.23	-0.61 ± 0.26	3.61	-1.85	5.59E-03	1.022	0.004
2-nonen-4-one	17.97	32064-72-5	0.44 ± 0.20	-0.63 ± 0.28	3.26	-1.71	3.33E-03	1.065	0.015
4-heptanone	11.08	123-19-3	-0.60 ± 0.42	0.86 ± 0.47	3.17	1.67	2.69E-02	1.446	0.004

RT = Retention Time; CAS No. = Chemical Abstracts Service registry number; VIP = Variable Importance in Projection; PLS = Partial Least Squares; RF = Random Forests

[a]All compounds were identified by spectral library search with match probability >70%

[b]Raw Fold change = abundance(condition A)/abundance(condition B)

[c]Log_2 Fold Change = log_2(abundance(condition A) – log_2(abundance(condition B))

In the Partial Least Squares-Discriminant Analysis (PLS-DA), the 28 VOCs were reduced to five PLS latent variables with fair discrimination ability between males and females ($Q^2 = 0.62$, permuted $P = 0.001$) (Figure 19). The first latent variable, accounting for 49.7% of the explained variance, corresponded to the date samples were run on. There was one set of samples from male SB#2660 that was run two weeks prior to all the other samples, and unfortunately this created a large batch effect that was difficult to overcome. In the design for Chapter 3, this was fixed through the retention time locking procedure to maintain more consistent retention times across batches and by running more samples over fewer days.

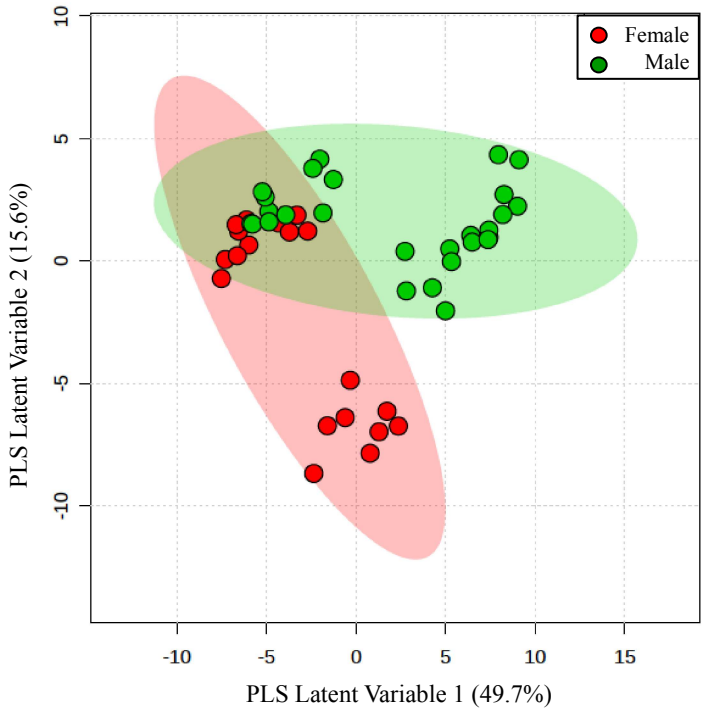


Figure 19. Scores on first 2 latent variables from partial least squares-discriminant analysis shown for female samples (n = 19 samples) in red and male samples (n = 27 samples) in green. Variance explained by each latent variable is shown in brackets.

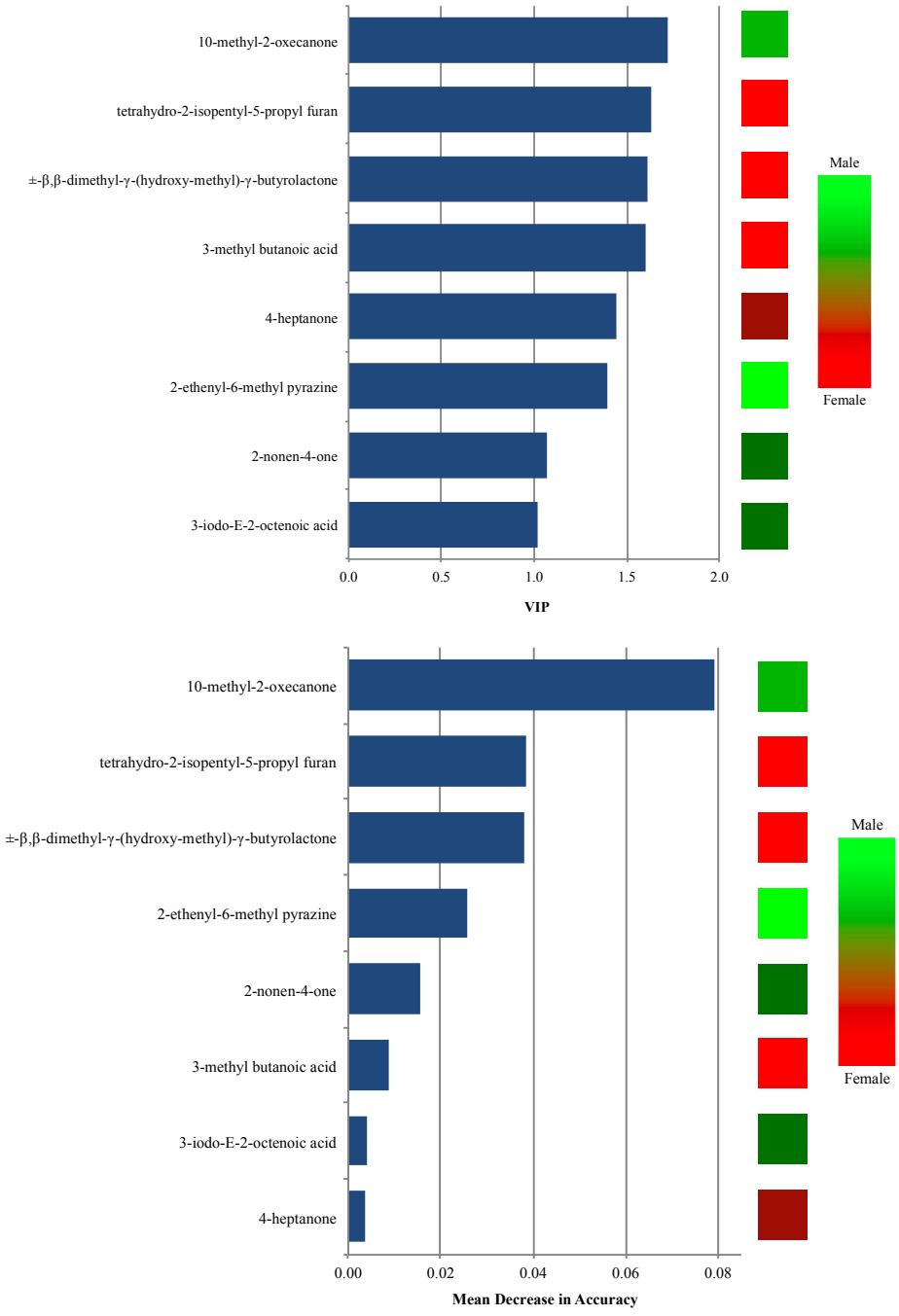


Figure 20. (a) Variable influence on projection (VIP) scores on partial least squares latent variable 1 and (b) mean decrease in classification accuracy for random forests model when each VOC is excluded for the top 8 most influential VOCs in a comparison between males (n = 27) and females (n = 19). Boxes on right indicate the relative abundance of each VOC from more abundant in males (green) to more abundant in females (red).

In the random forest (RF) model, 17/19 samples were correctly classified as female and 23/27 samples were correctly classified as male, resulting in an OOB error = 0.13. In both the PLS-DA and RF classifications, the three VOCs that contributed the most to the classification were 10-methyl-2-oxecanone, tetrahydro-2-isopentyl-5-propyl furan, and the butyrolactone (Figure 20).

Although this analysis did not directly reproduce the findings in Table 4 or Table 7, many of the same VOCs were found across these three analyses and there were no VOCs that contradicted findings of previous chapters. Overall, these results provide confidence in the findings from Chapter 2 Table 4 and Chapter 3 Table 7. These findings also suggest that the data processing and analysis methods used in Chapter 3 can be excellent tools for researchers wishing to study differential expression in urinary VOCs in mammals.

APPENDIX 4: MANED WOLF URINARY HORMONE ANALYSES

Introduction

To determine which maned wolf urinary VOCs are related to reproduction in Chapter 3, I wanted to correlate VOC abundances in urine with hormone concentrations in the same urine sample. Non-invasive fecal hormone monitoring is widely established in this species (Wasser et al. 1995; Velloso et al. 1998; Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a), but matched fecal samples were not available. Thus, I attempted to measure hormone metabolite concentrations in maned wolf urine. In one maned wolf male tested, 97% of radioactively labeled testosterone was excreted in feces as compared to urine (Velloso et al. 1998), but nevertheless, if it was possible to recover hormone concentrations from urine, this would be a powerful addition to the analyses of Chapter 3.

Methods

For each sample used in Chapter 3, enzyme immunoassays were run to assess urinary estrogen metabolite concentrations for female urine samples and urinary androgen metabolite concentrations for male samples. Hormone antibodies were obtained from Coralie Munro at the University of California, Davis, CA. For females, antibodies against estradiol (polyclonal antibody R4972; 1:10,000 working dilution), estrone conjugate (polyclonal antibody R522-2; 1:25,000 working dilution), and estrone sulfate (polyclonal antibody R583; 1:5000 working dilution) were tried and for males an

antibody against testosterone (polyclonal antibody R156/7; 1:50,000 working dilution) was tried.

Serial dilutions of urine from female SB#2945 during the date closest to breeding showed non-parallel displacement curves when compared to the standards for estradiol and estrone conjugate, so estrone sulfate was used (Figure 21). The estrone sulfate assay showed recoveries over 100% (range: 121% - 189%, mean: 140%) suggesting that there were molecules in the urine that bound to the estrone sulfate antibody with higher affinity than the hormone molecules themselves. The resulting linear equation was $Y = 1.21X + 15.06$ with an $R^2 = 0.995$ (Figure 22). A serial dilution of a pooled male urine sample showed a displacement curve parallel to the standards for testosterone (Figure 21). Recovery ranged from 23% – 105%, with an average of 65%, meaning that this assay underestimated the true amount of testosterone in the sample. The linear equation was $Y = 0.59X + 12.27$, $R^2 = 0.988$ (Figure 22). Urine samples were run undiluted on the estrone sulfate assay and diluted 1:2 on the testosterone assay. Assays were run as previously described for fecal extracts (Velloso et al. 1998; Songsasen et al. 2006; Johnson et al. 2014a).

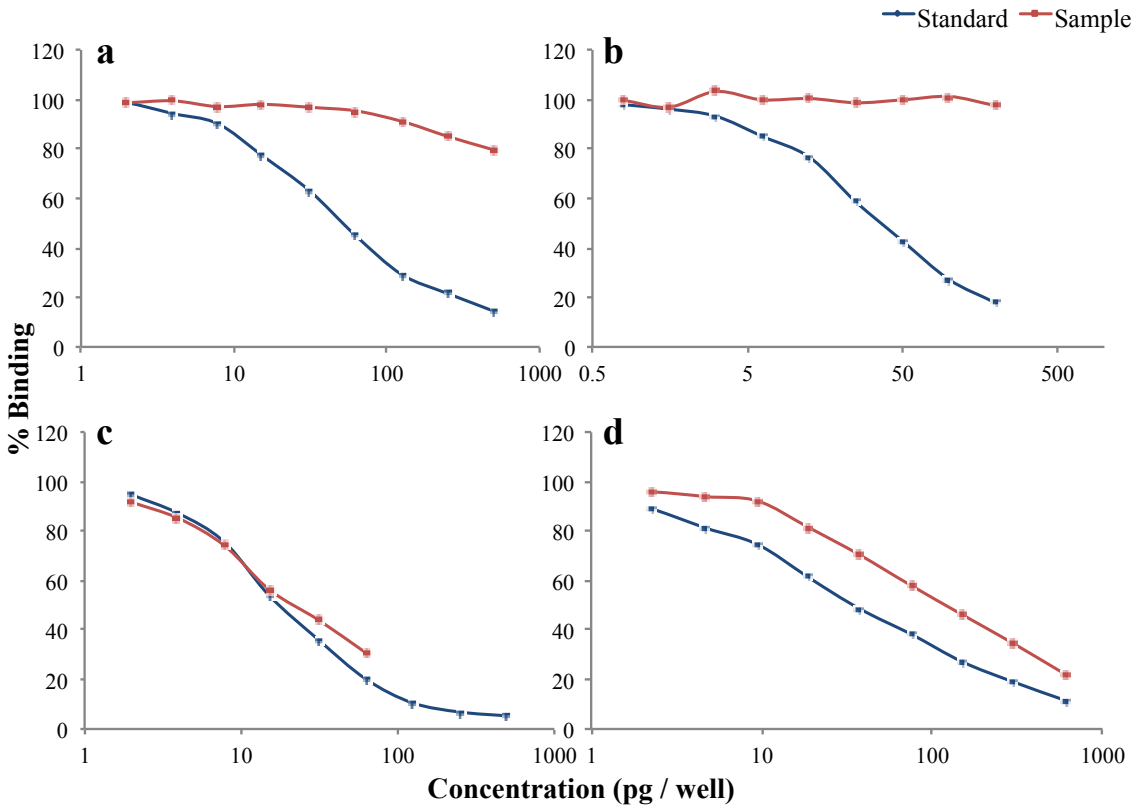


Figure 21. Comparison of urine hormone metabolite sample and standard curves for (a) estradiol and (b) estrone conjugate demonstrating a lack of parallelism, and for (c) estrone sulfate and (d) testosterone indicating parallel curves.

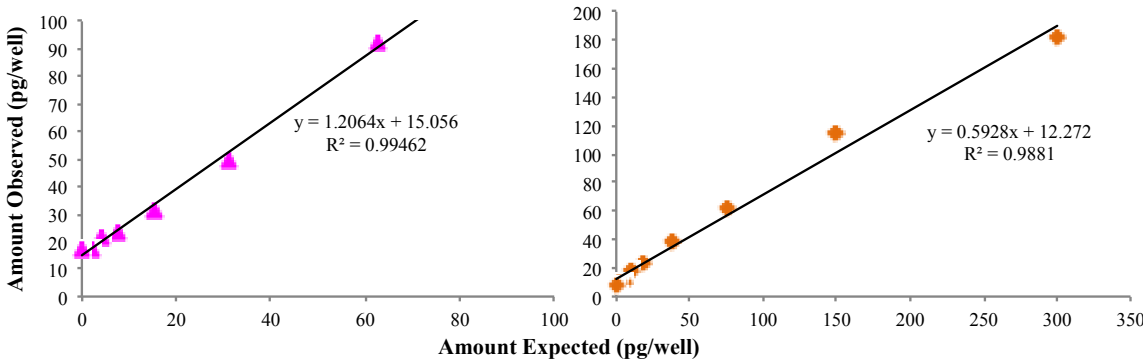


Figure 22. Recoveries for estrone sulfate (left) and testosterone (right) created by spiking standards into urine samples.

Urinary hormone concentrations were indexed with creatinine (Crt) to account for variations in water excretion. Thus, concentrations were expressed as hormone mass per milligram of Crt (Taussky 1954). Urine samples were diluted (1:50 in 0.2 M NaH2PO4, 0.2 M Na2HPO4, 0.2 M NaCl, pH 7.0) and added (0.05 mL) to a microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) along with Crt standard (0.00625 – 0.1 mg/mL; Sigma-Aldrich, St. Louis, MO) in duplicate. To each standard and sample, distilled H2O, 0.75 N NaOH, and 0.4 N picric acid were added (0.05 mL of each), and the assay was allowed to incubate at room temperature (25°C) for 30 min prior to assessing density (reading filter 490 nm, reference 620 nm) on a microplate spectrophotometer (Dynex MRX; Dynex Technologies, Inc., Chantilly, VA). Urine samples that were too dilute (<0.1 mg Crt/mL; <5% of samples) were not analyzed for hormone content and were discarded.

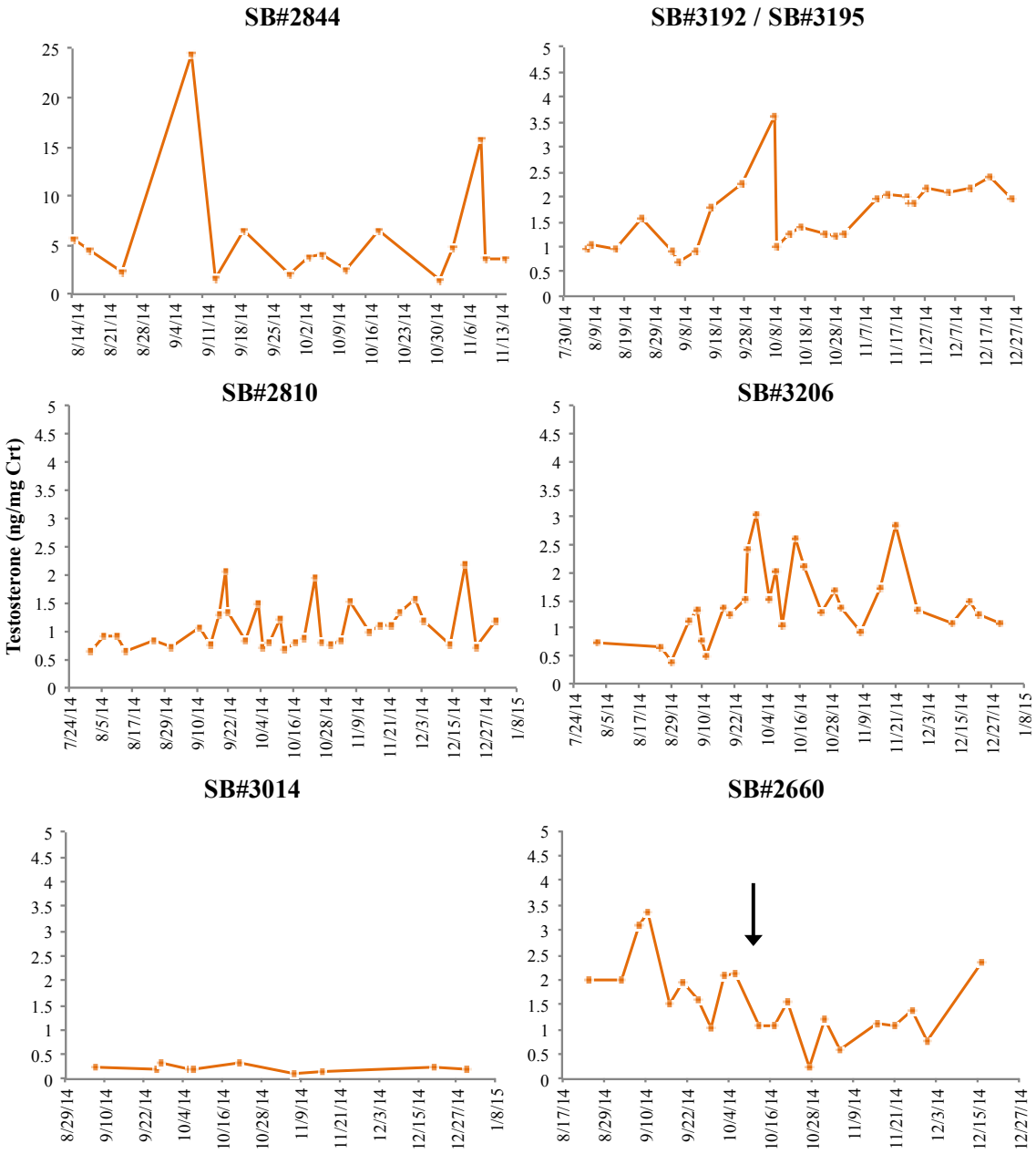


Figure 23. Urinary androgen metabolite levels indexed to creatinine for male maned wolves (n = 7). Collections were from 8/1/2014 – 12/31/2014, but no samples were collected in December for SB#2844. SB#2844 was housed with female #3184 but no breeding activity was seen, SB#3192 and #3195 were housed together, SB#2810 was housed singly, and SB#3206 was housed with his sister (spayed), SB#3014, a castrated male, was housed with female #2536, and SB#2660 was housed with female #2945 and bred 10/8/2014 (shown with arrow) and produced pups 12/12/2014.*Note y-axis scale is different for SB#2844. SB#2810 was on prednisone steroids during this period.

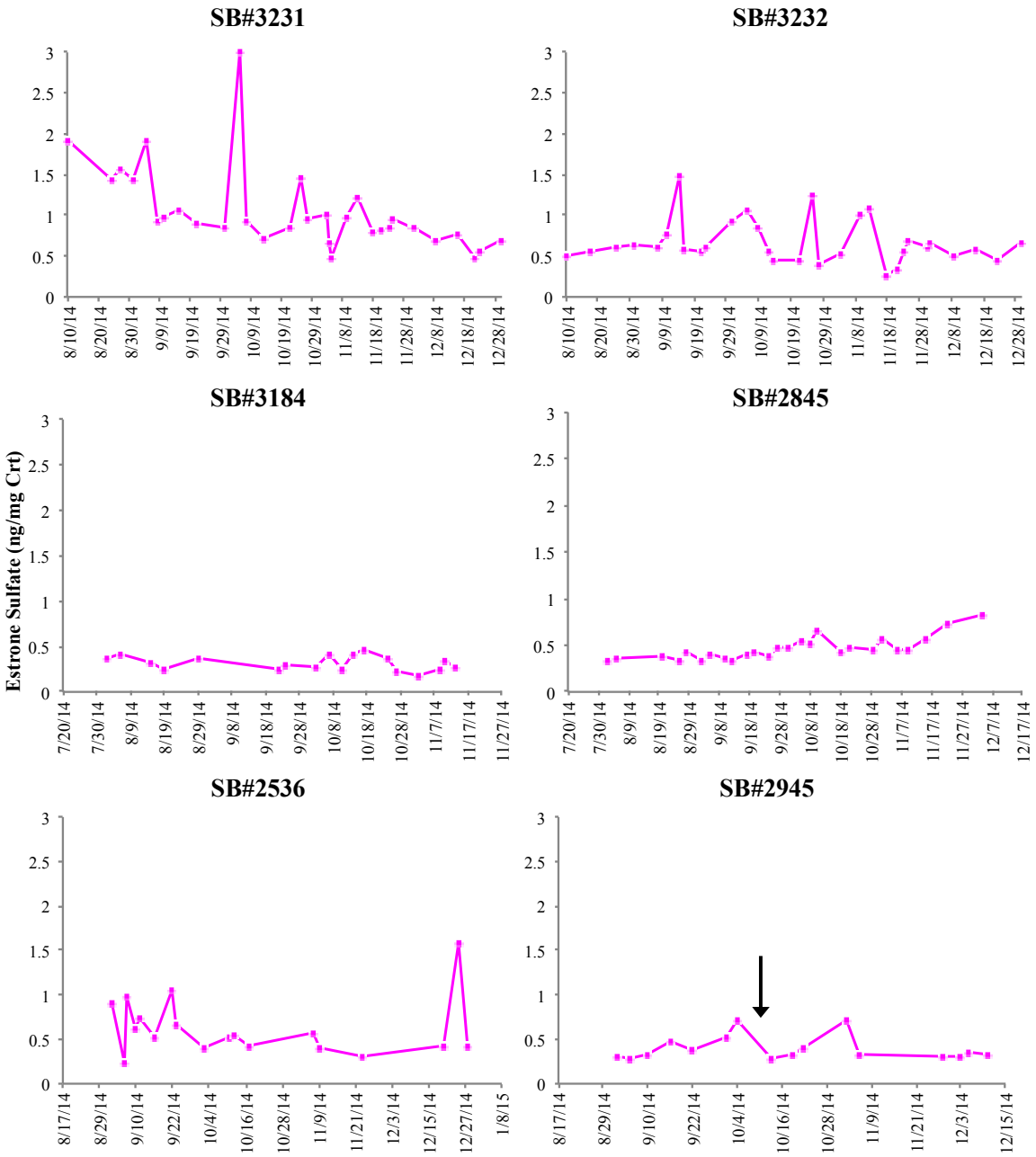


Figure 24. Urinary estrone sulfate levels for female maned wolves (n = 6).
Collections were from 8/1/2014 – 12/31/2014, but no samples were collected in December for SB#3184. SB#3231 and SB#3232 were housed together, SB#3184 was housed with male #2844 and no breeding was seen, SB#2845 was housed with her yearling pups, SB#2536 was housed with castrated male #3014, and female SB#2945 housed with male #2660 and bred 10/8/2014 (shown with arrow) and produced pups 12/12/2014.

Results and discussion

Despite reports that maned wolves excrete the vast majority (97%) of testosterone in feces (Velloso et al. 1998), the present results show that it is possible to measure testosterone levels in maned wolf urine. The castrated male, SB#3014, showed very low levels of the hormone throughout the breeding season while the intact males had higher, fluctuating levels (Figure 23). The male that bred during the 2014 season, SB#2660, showed a pattern consistent with that seen in fecal hormone studies and semen studies (Songsasen et al. 2014), where testosterone levels and sperm production are high leading up to breeding and then drop on the day of breeding (Figure 23).

The ability to detect estrogens in the urine is seemingly far less reliable, though the lack of frequent sampling could mean that an estrogenic peak would be occur between sampling dates. A radioactively labeled hormone infusion has not yet been conducted in females or with any other hormone besides testosterone, so it is not known if females metabolize steroid hormones similarly to males and thus, if we should expect the vast majority of hormone excretion in feces as opposed to urine. After parallelism analyses for estradiol and estrone conjugate showed an almost complete lack of parallelism, I opted for the estrone sulfate assay, which showed a curve relatively parallel to that of the standards. However, the high percent recovery (mean of 140%) for this assay suggested a problem. Potentially, there were molecules in the urine that bind to the estrone sulfate antibody at a higher affinity than the hormone molecules themselves.

None of the females showed discernable patterns similar to profiles seen with fecal hormone monitoring (Wasser et al. 1995; Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a). Even the female who bred during this season did not show any

characteristic rise in estrogens (Figure 24). Typically fecal samples for hormone monitoring are collected daily or every other day because female reproductive hormone peaks can be short lived (1 – 2 days). This female bred 10/8/2014 and samples were collected on 10/4/2014 and 10/13/2014. Thus, the estrogenic peak could have occurred between these two dates and resulted in no detectable peak on sampling dates.

Perhaps like male excretion of testosterone, female steroid hormones are mostly excreted in feces, making them more difficult to assess in urine. It is also possible that the estrone sulfate assay did not bind to the hormone molecules with high affinity, suggested by the high recovery values. To ascertain whether this was the case, High-performance liquid chromatography or liquid chromatography-mass spectrometry should be conducted for maned wolf urine.

For the above reasons, I elected not to correlate the abundances of urinary VOCs to urinary hormone levels. A follow up study should correlate urinary VOC abundances to hormone levels. However, using established fecal hormone monitoring methods would be preferable to attempting to measure steroid hormone concentrations in urine. As such, matched urine and fecal samples should be collected for use in future studies.

APPENDIX 5: MANED WOLF ETHOGRAM

Stimuli Trays (Figure 13**):**

1	– Farthest uphill. To the left and uphill of the upper yard teepee den.
2	– To the right of the upper yard teepee den.
3	– To the right of the igloo den near the fence to the lower yard.
4	– Outside of the lower building entrance in the lower yard. To the right of the guillotine gate between yards.
5	– Just uphill from the tree in the lower yard.
6	– Downhill of the tree in the lower yard.

Subject body state:

Not visible	– Cannot see subject to determine behavior
Inactive lie down	– Lying down, head down
Alert lie down	– Lying down, head up
Sit	– Rear end and front paws on ground
Stand	– All paws on ground
Walk	– Slow locomotion with two paws on ground at a time
Trot/Run	– Locomotion faster than walk

Proximity to conspecifics or nearest tray:

1	– Within one body length
2	– Between one and two body lengths
Far	– Farther than two body lengths

Event behaviors:

Focused attention	– Ears pointed towards interest, body tense
Sniff Air	– Nose higher than top of head, inhaling air
Sniff Object	– Nose pointed at object, inhaling***
Paw Object	– Paw contacting object (excludes ground)
Flehmen	– Mouth open, tongue out, nose wrinkled
Face/Neck Rub	– Contact of face, neck with ground or object
Urinate	– Urinating on ground (lasting longer than SM)
Scent mark	– Urinating on object to mark
Defecate	– Rounded body, defecating
Dig in substrate	– Using paws to move substrate
Other behavior	– No applicable code ***describe in margin**

Agonistic encounters:

Charge	– Move rapidly towards partner with ears back, head down, mane

piloerect, and then lunge towards partner with stiff forelegs

Bat – Stiff forelegs jab at partner's chest

Gape – Open mouth with lips pulled back, ears back, oriented towards partner

Crouch – Body held low to ground and usually curled, head flattened and turned to partner

***this was the only behavior to occur in response to urine scent stimuli

APPENDIX 6: CHEMICAL ANALYSIS OF URINE USED IN CHAPTER 4

Introduction

In Chapter 4, urine from male maned wolf SB#2844 was collected over 22 dates in October and November of 2010 and 2011. Exposure of three female maned wolves to this urine led to ovarian activity in SB#2539 similar to the early stages of breeding in paired females but no ovarian activation in the other two females, #2353 and #2354. The male urine was collected during peak breeding season dates; however, this male was not paired with a female during either of these years. Maned wolves are induced ovulators with the female only ovulating in the presence of a male. It is conceivable that a male may only produce certain reproductive signals when paired with a female, making the present urine from the unpaired male less attractive than comparable samples from a paired male. On the other hand, it is also possible that the male produces signals indicating his unpaired status as availability for pairing, making this male's urine more attractive to the female. While this study was not designed to answer that question, it is nevertheless important to investigate what VOCs possibly indicating pairing status the females in the Chapter 4 study were exposed to.

Methods

Following methods from Chapter 3, three replicates of the pooled urine from male #2844 were run through GC-MS. The data analysis pipeline from Chapter 3 was not used here. The present goal was to identify which VOCs the females were exposed to. Thus,

instead of the data analysis pipeline used in Chapter 3, these raw data files (Agilent .D format) were analyzed using MassHunter Quantitative Analysis software ver. B.07.00 (Agilent Technologies, Santa Clara, CA). The seven most abundant compounds from Table 5 and the six compounds that differed between paired and unpaired males (Table 9) were used to create a target list (Table 15). Using the MassHunter software, each replicate data file was searched for the peak area of each of the target analytes. Areas were $\log_2$ transformed and then averaged across the replicates and are reported as mean ± SEM. These values were then compared to the $\log_2$ transformed peak areas of the same target analytes in the samples from paired males and unpaired males from Chapter 3 using Analyses of Variances (ANOVAs). Results were considered significant at the 0.05 level.

Table 15. Compounds used in targeted analyte search.

Compound	RT	CAS No.	Quant Ion	Qual Ion 1	Rel. Abund.	Qual Ion 2	Rel. Abund.
2,5-dimethyl pyrazine	9.31	123-32-0	42	108	92.6%	81	14.7%
3-ethyl, 2,5-dimethyl pyrazine	11.57	13360-65-1	135	136	85.3%	56	26.4%
2-methyl-6-(1-propenyl) pyrazine	13.67	18217-81-7	133	134	83.0%	66	23.9%
2-Buten-1-ol, 3-methyl	9.25	556-82-1	71	41	53.6%	53	34.5%
3-Buten-2-ol, 2-methyl	4.49	115-18-4	71	43	55.3%	59	32.8%
4-heptanone	6.00	123-19-3	43	71	84.6%	114	14.3%
trimethyl pyrazine	10.72	14667-55-1	42	122	66.4%	81	16.3%
1,1'-thiobis-cyclopentane	14.55	1126-65-4	69	101	66.8%	68	51.0%
4-nonanone	9.33	4485-09-0	71	58	34.3%	99	34.2%
methyl ester benzoic acid	15.50	93-58-3	105	77	62.4%	136	38.5%
2-nonen-4-one	12.48	32064-72-5	69	84	47.0%	125	10.0%
2-acetyl-6-methyl pyrazine	16.66	22047-26-3	136	94	54.5%	93	28.9%
benzaldehyde	13.27	100-52-7	77	106	93.6%	105	88.9%

Quant = Quantitative
Qual = Qualitative
Rel. Abund. = Relative Abundance, the percentage of the quantitative ion peak

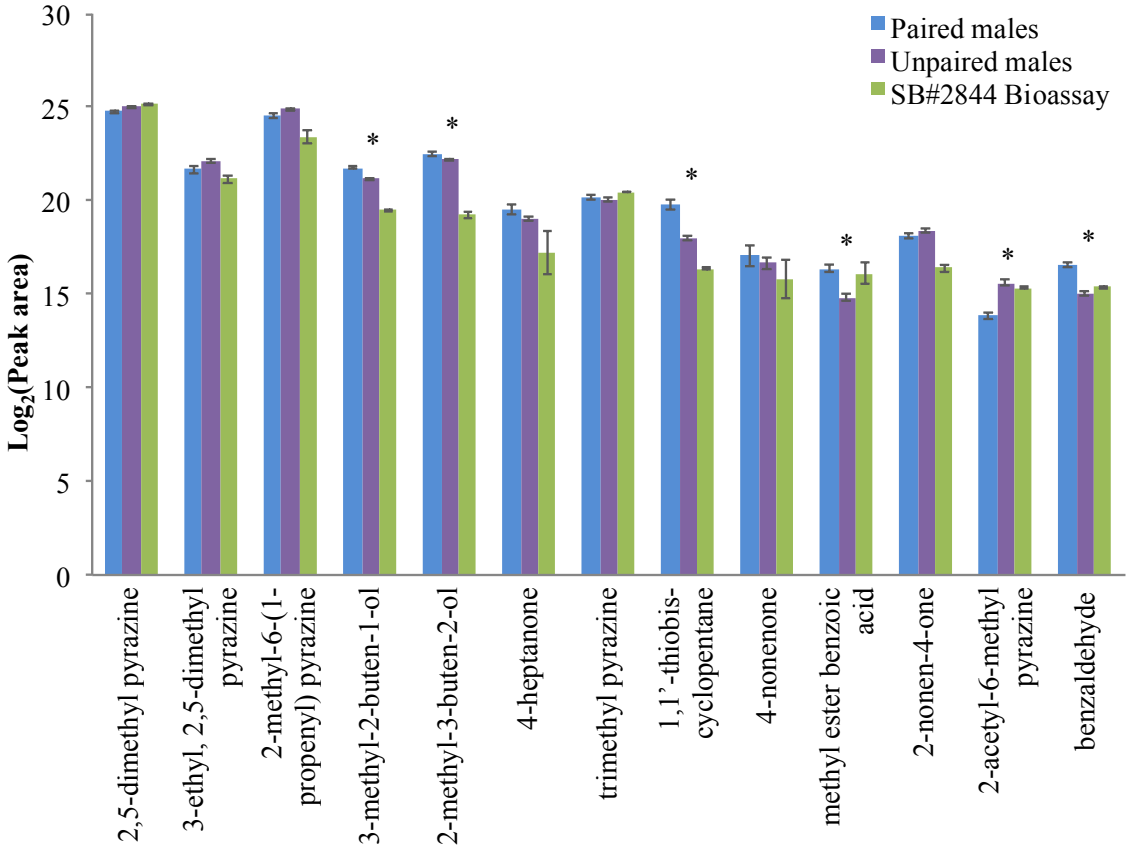


Figure 25. Peak areas log_2 transformed and compared across urine samples from paired males, unpaired males, and a sample from SB#2844 used in a behavioral bioassay in Chapter 4. The first seven compounds are those commonly found in maned wolf urine while the final six compounds are those that were found to differ by pairing status in Chapter 3. An asterix indicates a significant difference in an Analysis of Variance.

Results and discussion

The findings indicate that the sample from SB#2844 is more similar to those from unpaired males than from paired males. Levels of common maned wolf volatiles 2,5-dimethyl pyrazine, 3-ethyl, 2,5-dimethyl pyrazine, 2-methyl-6-(1-propenyl) pyrazine, 4-heptanone, and trimethyl pyrazine were not different between paired males, unpaired males, and the bioassay sample ($P > 0.05$) (Figure 25). The bioassay sample had lower levels of 3-methyl-2-buten-1-ol ($F_{2,280} = 13.45$, $P < 0.001$) and 2-methyl-3-buten-2-ol

($F_{2,280} = 14.31$, $P < 0.001$) compared to paired males and unpaired males meaning that this sample was more similar to those from unpaired males.

For compounds that were found in Chapter 3 to differ by pairing status, the bioassay sample showed lower levels than both paired and unpaired samples for 1,1'-thiobis cyclopentane ($F_{2,280} = 28.16$, $P < 0.001$), meaning that it was more similar to unpaired males (Figure 25). 4-Nonanone and 2-nonen-4-one showed no differences in peak area across groups ($P > 0.05$). For 2-acetyl-6-methyl pyrazine ($F_{2,280} = 23.82$, $P < 0.001$) and benzaldehyde ($F_{2,280} = 24.64$, $P < 0.001$), the sample from SB#2844 used for the bioassay was similar to the samples from unpaired males and those differed significantly from paired males. Methyl ester benzoic acid was the one compound that failed to follow the expected pattern where the bioassay sample was more similar to the unpaired male samples. For this compound, the bioassay sample and the paired male samples showed higher levels than the samples from unpaired males ($F_{2,280} = 15.02$, $P < 0.001$).

Overall, these data suggest that the sample provided to the females in the behavioral bioassay of Chapter 4 contained many common maned wolf VOCs at levels similar to those found for other males. Because levels of VOCs found to differ by pairing status were more similar to levels from unpaired males, there may be some indication to the females that male SB#2844 was unpaired. Therefore, repeating the bioassay with urine from a paired male remains an important way to understand more about the chemical signals present in maned wolf urine. Perhaps a male only produces certain reproductive signals when paired with a female, making unpaired male urine less

attractive to females than paired male urine. Alternatively, the unpaired male may produce signals indicating his availability for pairing making this male's urine more attractive to the female. A study designed to test these hypotheses would go a long way towards understanding reproductive signaling in the maned wolf.

APPENDIX 7: EFFECTS OF FREEZING AND AGEING ON MANED WOLF URINARY VOCS

Introduction

The maned wolf urine used throughout the studies of this dissertation was frozen as soon as possible after collection (within 8 h) and was maintained at -20°C until the day of processing and running through GC-MS. Human urinary VOCs do not change substantially as a result of freezing (Smith et al. 2008; Alwis et al. 2012), but no studies have analyzed changes in maned wolf urinary VOCs due to freezing. Many of the common maned wolf VOCs increase in abundance after ageing for multiple days at room temperature (Goodwin et al. 2013). Interestingly, centrifuging and filtering out microbes does not alter this effect, suggesting that microbial action is not involved in the increases (Goodwin et al. 2013). The present study was conducted to learn more about the effects of freezing and ageing on maned wolf VOCs.

Methods

One sample from male SB#2954 collected on November 25, 2016 (age = 9 years old) and one sample from female SB#3260 collected on December 2, 2016 (age = 4 years old) were used. On the day of collection, each sample was divided into 15 aliquots of 1 mL each. Following the methods in Chapter 3, ACS-grade NaCl was added to saturation along with an internal standard. Fresh samples were run through GC-MS in triplicate (0 h) and then frozen at -20°C for 24 h, 48 h or 72 h or aged at room temperature (24°C) for

24 h, 48 h, or 72 h (Figure 26). Due to a GC-MS machine malfunction, the 24 h data were unavailable for the male sample, so this sample was only analyzed at 48 h and 72 h.

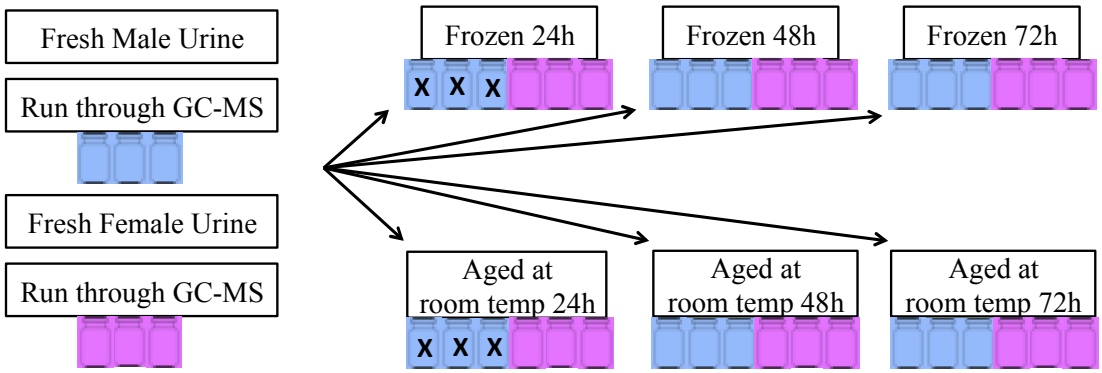


Figure 26. Schematic of methods to assess the effect of freezing and ageing on maned wolf urinary VOCs.

Table 16. Compounds used in targeted analyte search.

Compound	RT	CAS No.	Quant Ion	Qual Ion 1	Rel. Abund.	Qual Ion 2	Rel. Abund.
2,5-dimethyl pyrazine	9.31	123-32-0	42	108	92.6%	81	14.7%
3-ethyl, 2,5-dimethyl pyrazine	11.57	13360-65-1	135	136	85.3%	56	26.4%
2-methyl-6-(1-propenyl) pyrazine	13.67	18217-81-7	133	134	83.0%	66	23.9%
2-Buten-1-ol, 3-methyl	9.25	556-82-1	71	41	53.6%	53	34.5%
3-Buten-2-ol, 2-methyl	4.49	115-18-4	71	43	55.3%	59	32.8%
4-heptanone	6.00	123-19-3	43	71	84.6%	114	14.3%
trimethyl pyrazine	10.72	14667-55-1	42	122	66.4%	81	16.3%

Quant = Quantitative
Qual = Qualitative
Rel. Abund. = Relative Abundance, the percentage of the quantitative ion peak

Instead of the data analysis pipeline used in Chapter 3, these raw data files (Agilent .D format) were analyzed using MassHunter Quantitative Analysis software ver. B.07.00 (Agilent Technologies, Santa Clara, CA) with the goal of understanding how

maned wolf urinary VOCs change over time at two different temperatures. The seven most abundant compounds from Table 5 were used to create a target list (Table 16). Using the MassHunter software, each replicate data file was searched for the peak area of each of the target analytes. Areas were $\log_2$ transformed and then averaged across the replicates and are reported as mean ± SEM.

To enable direct comparisons to the findings of Goodwin et al. (2013), their data analysis procedure was also followed after averaging over replicates. Chromatographic peak areas were divided by the peak area obtained from the fresh sample. Thus, these results were reported as ratios of the fresh sample peak area.

Results and discussion

The $\log_2$ normalized abundances of all seven VOCs remained stable over 72 h at both 24°C and -20°C (Figure 27). These VOCs represented compounds from multiple classes: pyrazines, alcohols, and a ketone and none of them displayed any sensitivity to freezing or to leaving out at room temperature, supporting the conclusions of Alwis et al. (2012) and Smith et al. (2008) that urinary VOCs remain stable throughout the freeze-thaw process.

Several of the VOCs analyzed in the present study were the same VOCs analyzed by the Goodwin et al. (2013) study: 2,5-dimethyl pyrazine, 3-ethyl, 2,5-dimethyl pyrazine, trimethyl pyrazine, 2-methyl-6-(1-propenyl) pyrazine, 3-methyl-2-buten-1ol, and 2-methyl-3-buten-2-ol. Using the data analysis method used by Goodwin et al. (2013), the present study shows similar results for ageing the urine samples at room temperature (Table 17). For all of the VOCs except 2-methyl-3-buten-2-ol, the peak areas

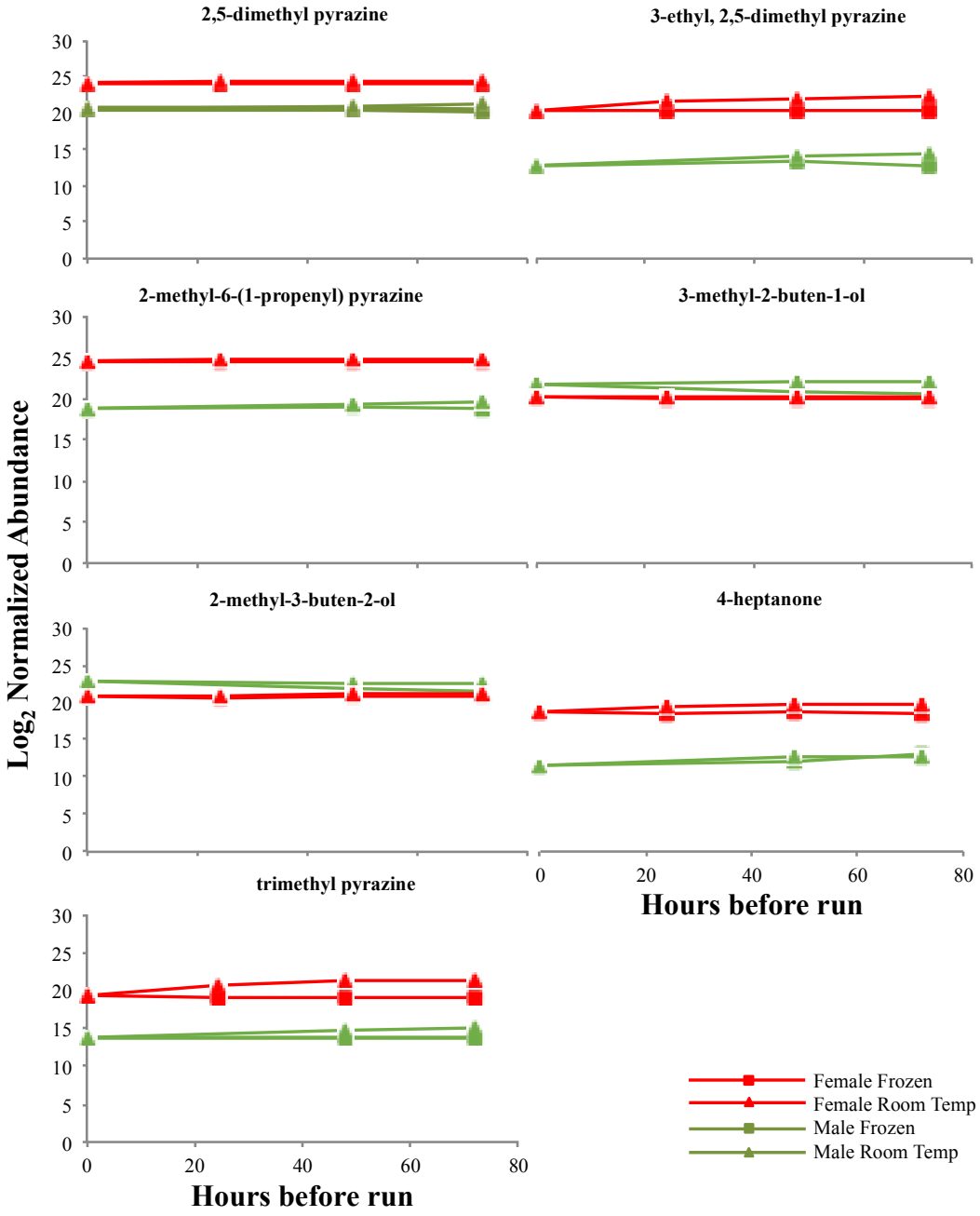


Figure 27. Log_2 normalized abundance of the seven most abundant maned wolf urinary VOCs over 72 h frozen or at room temperature.

Table 17. Ratio of chromatographic peak area to that of the fresh sample (0 h) for the seven most abundant maned wolf urinary VOCs.

Male					
	0 h	Frozen		RoomTemp	
Compound		48 h	72 h	48 h	72 h
2,5-dimethyl pyrazine	1.00	0.96	0.83	1.32	1.43
3-ethyl, 2,5-dimethyl pyrazine	1.00	1.63	0.96	2.22	3.04
2-methyl-6-(1-propenyl)-pyrazine	1.00	1.09	0.97	1.43	1.69
2-Buten-1-ol, 3-methyl	1.00	0.61	0.48	1.32	1.31
3-Buten-2-ol, 2-methyl	1.00	0.50	0.40	0.90	0.91
4-heptanone	1.00	1.41	3.31	2.37	2.55
trimethyl pyrazine	1.00	1.07	0.89	1.86	2.28

Female						
	0 h	Frozen			RoomTemp	
Compound		24 h	48 h	72 h	24 h	48 h	72 h
2,5-dimethyl pyrazine	1.00	0.97	0.92	0.91	1.06	1.11	1.14
3-ethyl, 2,5-dimethyl pyrazine	1.00	0.99	1.00	1.02	2.21	2.98	3.56
2-methyl-6-(1-propenyl)-pyrazine	1.00	0.93	0.93	0.94	1.12	1.15	1.21
2-Buten-1-ol, 3-methyl	1.00	0.91	0.88	0.87	1.03	1.07	1.07
3-Buten-2-ol, 2-methyl	1.00	0.80	0.95	0.94	0.97	1.14	1.16
4-heptanone	1.00	0.88	1.03	0.93	1.63	2.02	2.27
trimethyl pyrazine	1.00	0.92	0.88	0.93	2.85	3.96	4.53

increased after days of ageing at room temperature. 2-Methyl-3-buten-2-ol showed an initial loss in peak area (to male: 90% or female: 97% of fresh peak area) as a result of ageing followed by gains up to 91% (male) and 116% (female) of fresh peak area (Table 17). In almost all cases, freezing resulted in smaller peak areas than preserving at room temperature. The exception was 4-heptanone where freezing increased peak areas in the male sample and peak areas remained stable in the female sample.

It should be noted that neither the extraction technique performed for the present study (headspace SPME) nor the SPDE (solid phase dynamic extraction) sampling performed in the Goodwin et al. (2013) study allow for absolute quantitation. Calibration curves would nee to be created for each one of these compounds where an authentic

standard was available. By comparing the unknown amount to the calibration curve, a much more accurate absolute quantitation could be obtained in future studies.

The conclusions drawn from the raw peak area results and those from the $\log_2$ normalized abundances were different. Although the raw peak areas changed with freezing and with ageing, this effect was erased by the $\log_2$ normalization. For the sake of comparison with findings from Goodwin et al. (2013), raw peak areas were presented here. However, normalization procedures represent crucial pre-processing steps before meaning could be derived from the more complex analyses presented throughout the rest of the dissertation. Data normalization was critical for downstream metabolomics analyses presented in Chapters 2 and 3. Normalization procedures ensure that a fold change in abundance observed for a VOC with low abundance is as reliable as it is for one with high abundance (van den Berg et al. 2006; Kohl et al. 2012). VOC abundance variance differs greatly. This can have a biological reason as some VOCs show large abundance shifts without any biological effects, while others are more tightly regulated. However, differences in VOC variance can also have technical causes; relative measurements of low abundance VOCs are less precise than those of high abundance VOCs (van den Berg et al. 2006; Kohl et al. 2012). Although analyzing the raw chromatographic peak areas made good sense for this small-scale, simple study, this is neither practical nor advisable for the data analysis pipelines of Chapter 2 and 3.

Given the above results, the amount of time a sample remained frozen prior to running on GC-MS and the time after urination before sample collection each had little impact on the compound area. Compound areas remained stable over the timespan of

multiple days, but the effects of freezing samples for multiple weeks (as was the case for the samples in this dissertation) remain unknown.

While it is preferable to run samples as soon as possible after collection, running the samples fresh was logistically challenging. Future wildlife semiochemical studies should seek to minimize the time between urination, freezing, and running on GC-MS, while understanding that urine collection from wildlife held at zoological institutions already represents a significant time commitment from animal care personnel. These results show that normalization procedures used as a pre-processing step before metabolomic differential analyses can account for variable ageing and freezing times, allowing a more clear assessment of which urinary VOCs are differentially expressed across sexes, ages, reproductive seasons, health status, among other variables.

APPENDIX 8: BEHAVIORAL METHODS PILOT STUDIES

Introduction

In other mammal species, individuals are able to distinguish between conspecific urine collected during breeding season versus outside of breeding season, suggesting there are seasonal differences in urinary volatile composition (elephants: Slade et al. 2003; Bagley et al. 2006; Meyer et al. 2008, ferrets: Zhang et al. 2005, pandas: Swaisgood et al. 2002). Similar results have been found in canid species such as the gray wolf and the red fox. Whitten et al. 1980b presented a cocktail of synthetic urinary volatiles to red foxes, resulting in scent marking behavior. Raymer et al. (1986) found that administration of testosterone to castrated male gray wolves induced the production of urinary volatiles usually associated with intact males, further supporting the idea that urinary compounds reflect reproductive status in canids. Although no studies have been undertaken to examine this topic in maned wolves, previous research has shown that as breeding season approaches, maned wolves increase their sociality and the frequency of urine scent marking increases (Brady and Ditton 1979; Rodden et al. 1996; Jácomo et al. 2009).

There is evidence to suggest that maned wolves are induced ovulators (Songsasen et al. 2006; Reiter 2012; Johnson et al. 2014a), likely through olfactory signaling. 2,5-Dimethyl pyrazine composes a substantial portion of the volatile compounds of maned wolf urine, and is one of the main constituents contributing to maned wolf urine's

pungent odor (Childs-Sanford 2005; Goodwin et al. 2013). This compound is also a constituent of African wild dog (*Lycaon pictus*) feces but not urine (Apps et al. 2012), and is an alarm and trail pheromone in many species of insects (Guilford et al. 1987; Brophy 1989; Woolfson and Rothschild 1990).

No research has been conducted regarding the role of chemical signaling in maned wolves. Accordingly, this dissertation seeks to fill that knowledge gap. The goals of the pilot studies were to assess: (1) The role of 2,5-dimethyl pyrazine in maned wolf signaling; (2) Various methods of scent presentation; (3) An appropriate control scent, and (3) Behavioral responses of maned wolves to breeding versus non-breeding season urine.

Effects of 2,5-dimethyl pyrazine

Introduction

During the breeding season of 2009 (September - November), the behavioral and physiological effects of exposure to 2,5-dimethyl pyrazine were investigated. The goal of this study was to determine whether: (1) Maned wolves interact preferentially with 2,5-dimethyl pyrazine over other scents; (2) Lemon essential oil is an appropriate control substance for olfactory studies in this species; and (3) Maned wolves show a physiological response to scent presentation via fecal glucocorticoid or gonadal hormone metabolite levels. Lemon essential oil was selected as a possible control substance because it is non-toxic and easily available.

Methods

Five maned wolves, all housed at the Smithsonian Conservation Biology Institute were used. Three female wolves, SB#1818 (age 14), SB#2348 (age 8), and SB#2612 (age

6) were included along with two males, individuals SB#2611 (age 6) and SB#3120 (age 2). SB#1818 and SB#2348 had successfully reproduced in prior years. A fence line was shared between wolves SB#1818 and SB#3120 and between SB#2348 and SB#2611. Descriptions of the enclosures and diet can be found in Chapter 4. All procedures were reviewed and approved by the SCBI Institutional Animal Care and Use Committee (IACUC).

The study was divided into five parts: (1) Pre-exposure: 10 days of behavioral observation without exposure to scents; (2) Choice period: ten days of behavioral observation during exposure to four scents simultaneously (male urine, female urine, pyrazine, and lemon as control); (3) Opposite sex urine exposure: four consecutive days of exposure to opposite sex urine; (4) Pyrazine exposure: four consecutive days of exposure to 2,5-dimethyl pyrazine; and (5) Post-exposure: five days of no exposure to scents. Fecal samples were collected daily for the duration of the study.

Stimuli were prepared by soaking a piece of sterile gauze in each scent and placing it into a PVC tube (2.5 x 12 cm) with ten holes (0.5 cm diameter each). PVC tubes were fitted with caps on both ends and on one end, a short length (10 cm) of metal chain with a metal carabineer clip to enable attachment to the fence (Figure 28). Scents were: male urine collected during the breeding season of 2006 from one wolf, female urine collected during the breeding season of 2004 pooled from three wolves, 0.001% (v/v) 2,5-dimethyl pyrazine (Sigma-Aldrich, St. Louis, MO) in water, and 0.5% (v/v) lemon essential oil (Aura Cacia, Urbana, IA) in water. During the choice period, scent tubes were hung on the fence 2 m apart at wolf head height (~1 m) at the start of the

observation period. The order of the scents was determined using random sampling and was altered each day. Scent tubes were removed following the observation session, gauze was discarded and the tube was cleaned with soap and water. During the exposure periods, one scent tube of the given scent was hung on the fence from sunrise to sunset. Each evening the scent tube was cleaned.

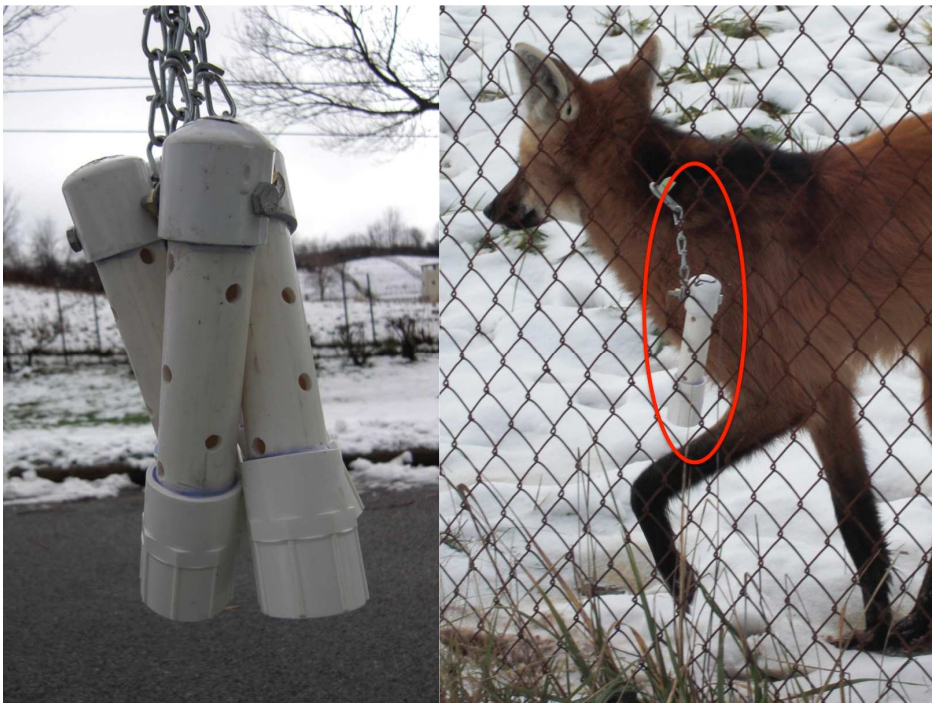


Figure 28. Scent tubes made from PVC tubing with ten holes for scent diffusion. Tubes were capped on each end and fitted with a short section of chain and a metal carabineer to enable attachment to the enclosure fence.

Quantitative behavioral data were collected on each wolf each day for the pre-exposure and the choice periods. Observations were 60 min within two hours of dawn or dusk, by the same observer using the focal animal method (Martin and Bateson 2007). Activity level was calculated by recording body state each minute (Appendix 5: Maned

wolf ethogram). "Active" body states were "run", "walk", "stand", "sit", and "alert". "Inactive" and any interval of "not visible" longer than 3 min were classified as an "inactive" body state. All occurrences of interactions with scent tubes ("sniff object", "sniff air", "paw object", "scent mark") were recorded as well (Appendix 5: Maned wolf ethogram). Daily activity rates were calculated by dividing the time wolves were active by the total observation time (60 min). For each session, the proportion of interaction with each scent tube was calculated by dividing the occurrences of interactions with each scent by the total minutes visible.

Fecal samples were processed and analyzed for hormone metabolite concentrations using the methods detailed in Chapter 4. Fecal cortisol metabolites (FCM) and fecal estrogen metabolites (FEM) were monitored for female maned wolves and FCM and fecal androgen metabolites (FAM) were monitored for males. Average extraction efficiency was 78% with a coefficient of variation <10%. FCM, FEM, and FAM were quantified by enzyme immunoassay using cortisol (R4866; 1:8500), estrone conjugate (R522-2; 1:25000), and testosterone (R156/7; 1:7500) antibodies (University of California, Davis, CA). Inter- and intra-assay variations were <10%.

To compare activity rates across the pre-exposure and choice periods, paired *t*-tests or *U*-tests were used. To assess scent tube preference, females and males were evaluated separately. The proportion of interactions with each scent tube was analyzed for differences using a Kruskal-Wallis.

Due to the small sample size (n = 5) and highly variable hormonal responses across wolves, fecal hormone metabolite profiles were analyzed separately for each

individual. Fecal hormone concentrations are reported as mean ± standard error (SEM). To determine the physiological response to the choice period, the change in baseline cortisol level was analyzed via paired *t*- or *U*-tests comparing the pre-exposure period to the choice period. Baseline cortisol values were calculated via an iterative process whereby values exceeding the mean + 1.5 standard deviations (SD) were excluded (Brown et al. 1994; Brown et al. 2016). The mean was then recalculated and the exclusion process repeated until no values exceeded the mean + 1.5 SD. To determine the wolves' physiological responses to the opposite sex urine scent and to the pyrazine scent, average levels of FCM, FEM, and FAM during the exposure periods were compared to the pre-exposure period via a one-way ANOVA on ranks.

Results and discussion

For all individuals, the presence of scent tubes significantly increased maned wolf activity level during the choice period as compared to the pre-exposure period, $t_4 = 3.09$, $P < 0.05$ (Figure 29). This suggests that maned wolves found these scents to be enriching, spending more time engaging with their environment than during the pre-exposure period (Clark and King 2008).

Baseline FCM were not altered during the choice period as compared to the pre-exposure period for wolves SB#2348, SB#2612, and SB#3120. SB#2611 showed a significant decrease in baseline FCM from the pre-exposure period (3016.92 ± 408.76) to the choice period (1161.93 ± 147.24; $U_1 = 12.00$, $P < 0.05$) while SB#1818 showed the opposite effect, exhibiting a significant increase in FCM from the pre-exposure period (283.35 ± 55.88) to the choice period (981.76 ± 76.89; $t_9 = 6.57$, $P < 0.05$). Individuals

may experience the scents in different contexts, based on their age, reproductive status, and other factors. For example, SB#1818 was elderly and perhaps exposure to urine from younger, reproductive individuals was perceived as threatening. Another possible explanation is that this wolf was very accustomed to her routine and experienced neophobia (Clark and King 2008) in response to the novel scent exposure.

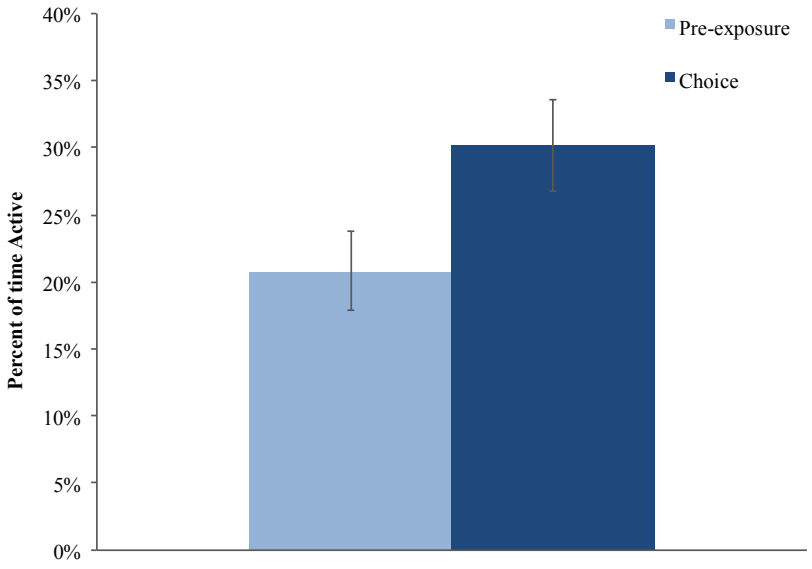


Figure 29. Percent of time spent active for five maned wolves during pre-exposure period and choice period when wolves were exposed to four scents simultaneously.

Table 18. Total number of occurrences of behaviors in response to scent stimuli for five maned wolves over ten hours of observation.

	Females (n = 3)			Males (n = 2)		
	Sniff Air	**Sniff Object**	**Scent Mark**	**Sniff Air**	**Sniff Object**	**Scent Mark**
Lemon	1	22	0	2	35	2
2,5-Dimethyl Pyrazine	0	9	2	1	20	2
Same Sex urine	0	15	0	3	22	6
Opposite Sex Urine	0	24	1	4	16	5

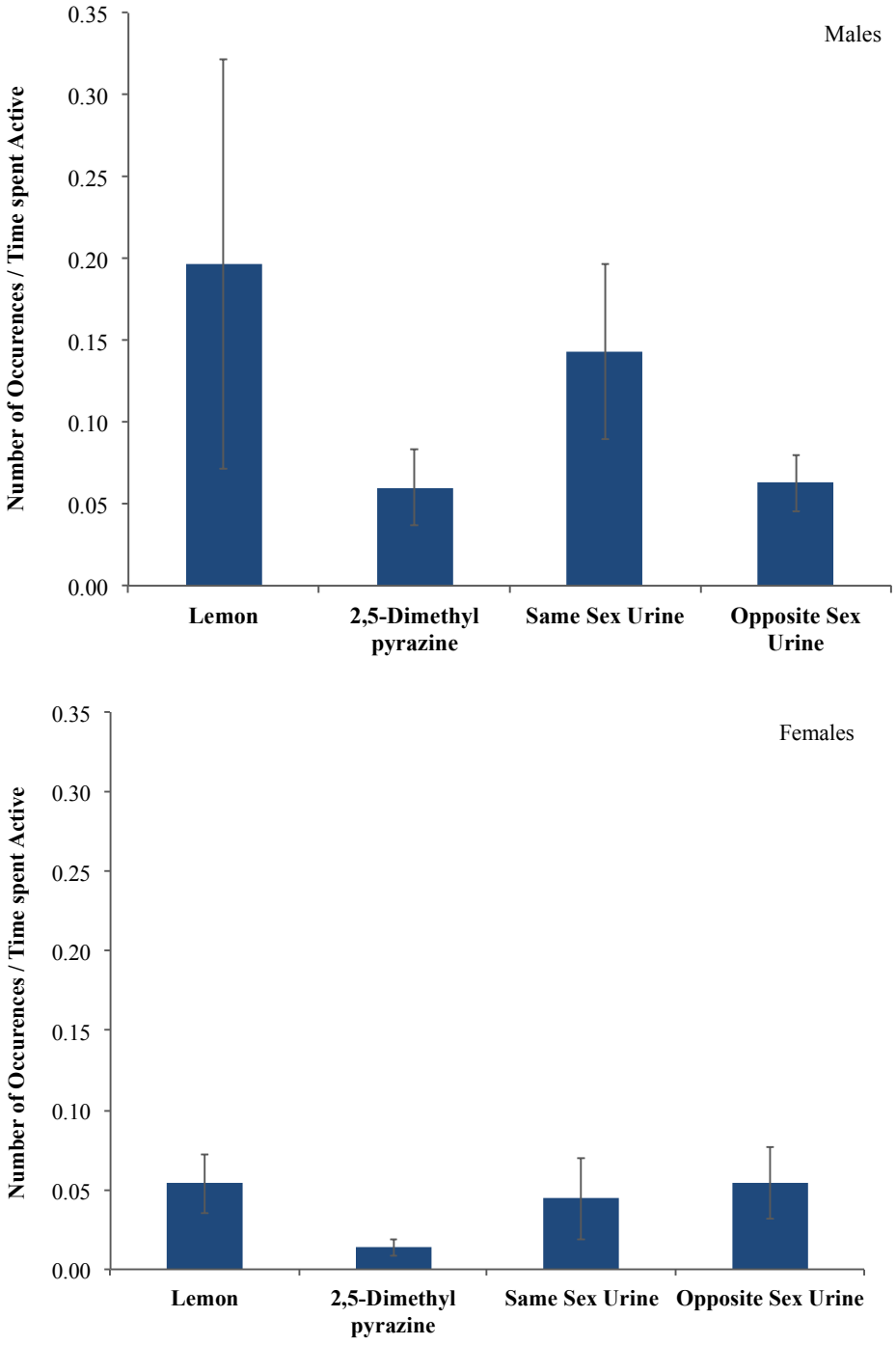


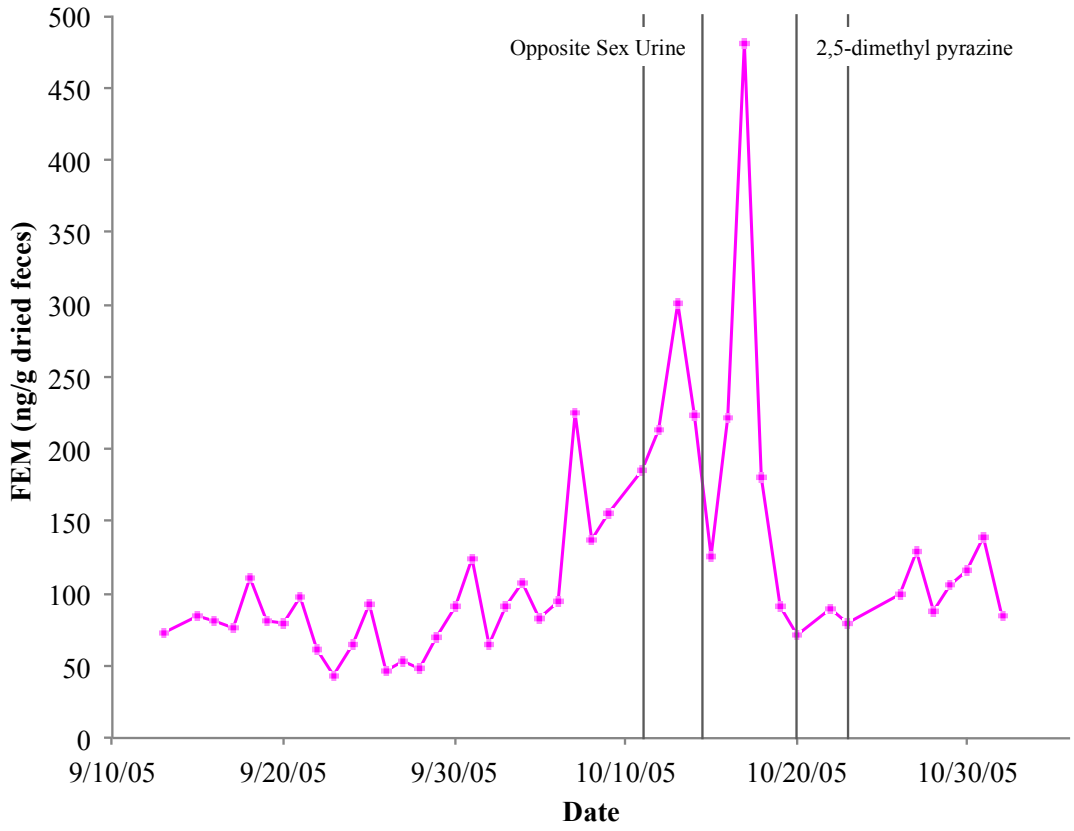
Figure 30. Mean proportion of interactions (number of occurrences / time spent active) with each scent for male (n = 2) and female (n = 3) maned wolves.

No significant preference for interacting with a particular scent was found for males ($H_3 = 4.35$, $P = 0.226$) or for females ($H_3 = 5.85$, $P = 0.119$; Figure 30). However the "scent marking" behavior was most common in response to urine over the other scents (Table 18). Therefore, I believe that there is a differential response in maned wolves to these different scents but that the sample size (number of hours of observation and thus the number of occurrences of scent marking) was too small to enable a detectable difference. Responses did not differ statistically between males and females ($\chi^2_3 = 1.65$, $P > 0.05$), also possibly due to small sample size.

Maned wolves seemed interested in consumption of the lemon scent, often licking the scent tube after performing "sniff object". We were hoping to identify a control scent that generated some interest at first due to novelty but subsequently showed decreased interest with habituation. Based on the sustained interest seen in this pilot study in response to lemon, I suggest trying a different scent as a control.

Reproductive hormone profiles showed interesting patterns for one pair of wolves sharing a fenceline, SB#2348 and #2611. Concentrations of FEM or FAM were higher during presentation of the opposite sex urine than during the pre-exposure period for both the male and female of this pair, but the differences were not statistically significant (Figure 31). It is unlikely that exposure to opposite sex urine for a few hours daily for four days prompted this hormonal response, though the timing of the hormone peaks directly following exposure to opposite sex urine is certainly intriguing. The ability of this pair to see and smell one another likely also stimulated release of reproductive hormones, leading to profiles similar to those of breeding pairs in full contact with each

other. This case is especially interesting due to the implications regarding the mechanism behind induced ovulation. It would seem that physical contact between the individuals of the pair is not necessary to induce ovarian activity but that olfactory contact is sufficient. Chapter 4 was specifically designed to test this hypothesis based on these findings.



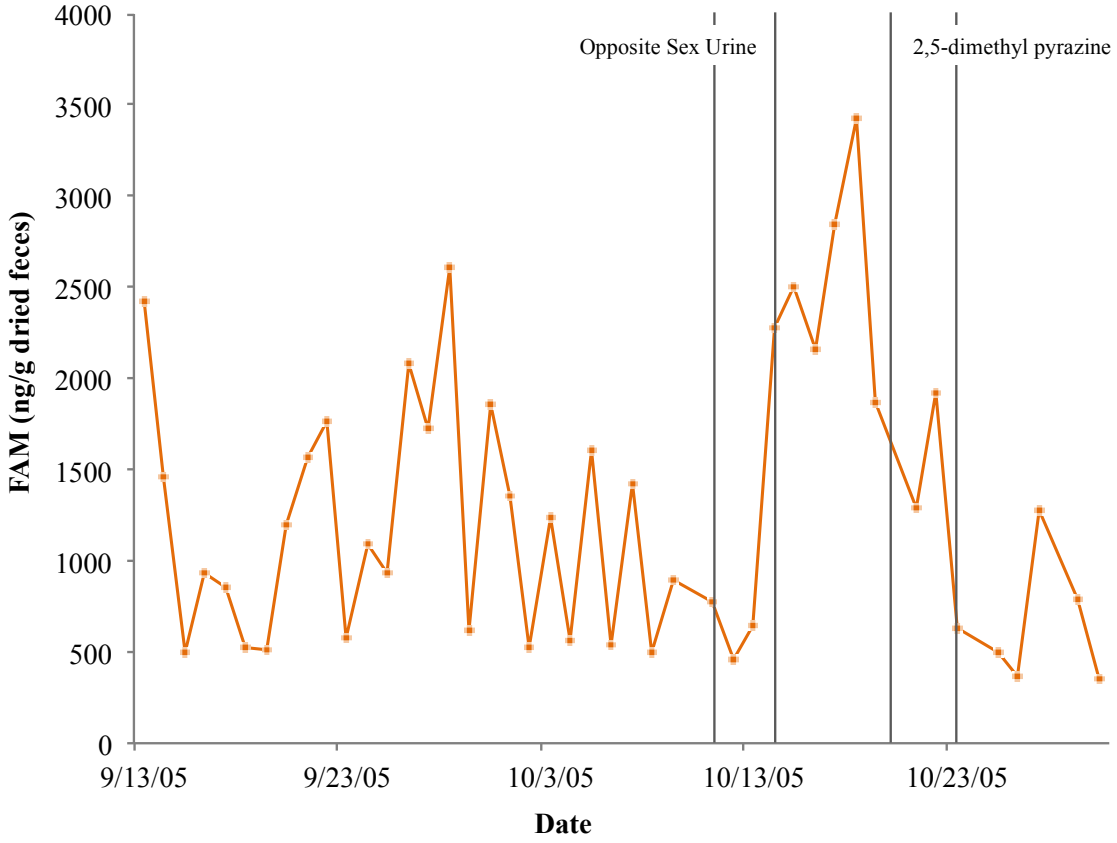


Figure 31 (a) Fecal estrogen metabolite (FEM) concentration (ng/g dried feces) for female SB#2348 and (b) fecal Androgen Metabolite (FAM) concentration (ng/g dried feces) for male SB#2611 who were housed sharing a fenceline. Periods of daily exposure to opposite sex urine and to 2,5-dimethyl pyrazine are shown with vertical lines.

In considering the experimental design in the present study, PVC is much too porous to provide a clean vehicle for scent presentation. When scent-soaked gauze is in contact with the PVC surface, no doubt odor molecules adhere to the surface and are retained in the PVC even after cleaning with soap and water. Therefore, a disposable method of bioassay scent presentation was tested in the following pilot studies.

Using vanilla as a control scent

Introduction

Based on the results from the 2009 pilot study, a study was conducted in 2011 to test vanilla extract as a control scent for use in future semiochemical research with the maned wolf. In the 2009 study, maned wolves were as interested in the lemon scent as in conspecific urine collected during breeding season, and maned wolves seemed to perceive lemon as a food item, often licking and biting the scent tube. Vanilla was selected due to it being a novel odor that lacks any signaling role. It is also easily available and safe for maned wolves to contact. Vanilla has been used as the control scent in chemical signaling bioassays with other mammals (Slade et al. 2003; Bagley et al. 2006; Schulte et al. 2007; Meyer et al. 2008). Interest in vanilla was compared to a positive control, opposite sex breeding season urine, and a negative control, water. If vanilla extract is an appropriate control scent, I expected to see decreased interest over sessions in response to this scent compared to sustained, high interest in breeding season urine throughout the sessions, and low interest in water.

Methods

Nine maned wolves housed at the Smithsonian Conservation Biology Institute were used for this study from March 1, 2011 through May 17, 2011. Females were SB#2348 (age ten), #2612 (age eight), #2613 (age eight), #2539 (age seven), and #3184 (age one). Males were SB#3120 (age three), #2844 (age five), #2815 (age six), and #2374 (age ten).

Pine construction shims (sticks) were used for scent presentation and were discarded after each use. Sticks had a hole drilled through the top so that it could be

attached to the maned wolf enclosure fence using a metal carabineer (Figure 32). The behavioral observer was blind to the scent identity of the sticks so that observations would be unbiased. Thus, sticks were labeled with A, B, or C and were soaked by another researcher in opposite sex breeding urine diluted 1:2 in deionized water, vanilla diluted 1:174 in deionized water, or deionized water for 2 min. Sticks were then frozen until use in behavioral bioassays. Urine was collected from males (SB#2844, #2374, and #2814) during previous breeding seasons (September – February) and pooled together for presentation to females. Urine from females (SB#2348 and #2845) collected during the same months of previous years was pooled for presentation to males.

Figure 32. Pine construction shim used for bioassay scent presentation. Stick has hole drilled at the top to enable attachment to enclosure fence with metal carabineers.

Five behavioral observation sessions were conducted for each individual. Sessions were held before the morning feeding which was typically around 9:30AM or after the afternoon feeding at 4:30PM. Beginning 30 min before the session, the observer defrosted one stick of each scent. Sticks were hung on the maned wolf enclosure fence 2 m apart and at 1 m high in a randomized order for each session. Using a video camera, subject interactions with the scent sticks were recorded for 1 h (Appendix 5: Maned wolf ethogram). Sticks were then discarded at the end of the hour.

Videos were reviewed for the duration of interactions with each scent type. Average duration of time spent investigating each scent was assessed using *t*-tests and linear regression analysis. All results were reported as mean ± standard error of the mean (SEM).

Results and discussion

On average, maned wolves spent longer investigating opposite sex urine (6.07 ± 0.76 sec/h) than the water control (4.05 ± 0.36 sec/h, $t_{99.2} = -2.57$, $P < 0.01$) and marginally more time than the vanilla control (4.75 ± 0.47 sec/h, $t_{117.9} = -1.57$, $P = 0.059$). A linear regression for opposite sex urine showed that the interest remained constant over the sessions ($Y = -0.22X + 6.73$, $F_{1,65} = 0.24$, $P = 0.628$) while interest in vanilla significantly decreased over sessions ($Y = -0.64X + 6.72$, $F_{1,74} = 5.02$, $P = 0.028$). Interest in water was low, but steady ($Y = 0.17X + 3.50$, $F_{1,62} = 0.57$, $P = 0.451$) (Figure 33).

Interest in opposite sex breeding season urine remained high over all five sessions and interest did not decrease over time, suggesting that opposite sex breeding season urine may contain a semiochemical. Interest in vanilla, a novel scent, began high but

decreased over the five sessions. This decreasing interest suggests that vanilla is an appropriate control substance for semiochemical bioassays in the maned wolf.

At the beginning of the study scent sticks were hung on the inside of the maned wolf enclosure fence so that subjects could interact with the odor as much as possible. On three occasions, wolves bit the stick and on one occasion a wolf (SB#3120) consumed almost ¾ of the stick. After conversations with the vet staff, for the remainder of the study, scent sticks were hung on the outside of the fence to allow full olfactory contact but not physical contact with the sticks. This change prevented wolves from biting and chewing the sticks but did not seem to alter their ability to sniff and lick the sticks.

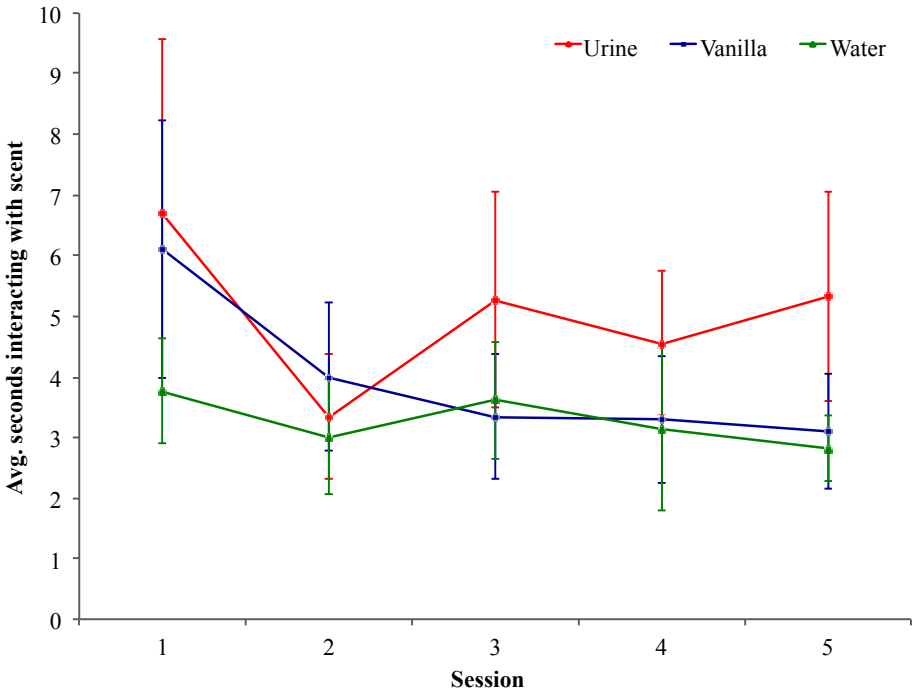


Figure 33. Average seconds spent interacting with three scents over five 1 h long sessions for nine maned wolves. Scents were opposite sex urine collected during breeding season, vanilla extract diluted 1:174 in water, and water.

Interest in breeding- versus non-breeding season urine

Introduction

Using the methods derived in the previous two pilot studies, a study was devised for the breeding season of 2012 to test whether females exhibit increased interest in male urine collected during breeding season compared with male urine collected during non-breeding season. If there are semiochemicals present in male urine that communicate reproductive readiness, I expected that the females would exhibit stronger interest in the urine collected during breeding season.

Methods

Two female maned wolves housed at the Smithsonian Conservation Biology Institute were used for this study: SB#2926 (age five) and #3184 (age two). These females were not recommended for breeding in 2012, so were available for this study.

Pine construction shims labeled A, B, or C were fully submerged in male breeding season urine, male non-breeding season urine, or a control (vanilla diluted 1:174 in deionized water) for 24 hours at room temperature. This process was conducted by a second researcher so that the behavioral observer would be blind to the scent identity of the sticks so as to remain unbiased. Urine for the breeding season scent was pooled from male SB#2844 for several dates in November 2011. Urine for the non-breeding season scent was pooled from SB#2844 (August 2012), #2611 (July 2006), and #2374 (summer 2007 and 2008). After soaking, sticks were frozen until use in the behavioral trials.

Sticks were defrosted 30 min prior to use and were hung on the outside of the enclosure fence at 2 m apart and 1 m high. The order of scent presentation was randomized for each session. Females were video recorded for 1 h sessions either before

morning feeding or after afternoon feeding. The goal was six successful sessions per subject. A session was deemed successful if the subject interacted with the scent stimuli at least once in the hour-long session. Sessions took place in September and October of 2012.

Videos were reviewed for the duration of all interactions (Appendix 5: Maned wolf ethogram) with each scent type. Due to low levels of interaction and only two subjects, summary statistics were reported but inferential statistical analyses were not conducted. Results were reported as mean ± standard error of the mean (SEM).

Results and discussion

Wolf #3184 was observed for a total of 11 sessions, and she interacted with at least one of the sticks in 7 of the sessions. For these successful sessions, this wolf was active (visible and awake) for an average of 10.86 ± 2.60 min. For three of the four unsuccessful sessions, SB#3184 was not visible at all through the whole hour session. Wolf #2926 was observed for 14 sessions and of those 6 were deemed successful observation sessions. For these successful sessions, this wolf was active (visible and awake) on average 27.67 ± 7.30 min. In five of the eight unsuccessful sessions, SB#2926 did not exit her den. Overall, activity levels were very low for these subjects, making it difficult to gather the amount of interaction data necessary to determine if maned wolves have a preference for one scent over the others.

In total the two females spent 12 sec sniffing vanilla, 32 sec sniffing male non-breeding season urine, and 43 sec sniffing male breeding season urine. There was one instance of SB#3184 licking a vanilla stick. SB#3184 performed scent marking within 30

sec of sniffing a stick on four occasions, twice in response to non-breeding season urine and twice after sniffing breeding season urine. This female also neck rubbed the fence under the breeding season urine three times and never performed this behavior near the other scents. Neck rubbing is a known maned wolf scent marking behavior (Rodden et al. 1996), but was never observed in other studies within this dissertation. SB#2926 never scent marked during an observation session.

It is clear that the urine scents were more stimulating than the vanilla control, but this study lacked the power necessary to determine the difference in interest level between breeding- and non-breeding season urine.

Conclusions

Collectively, these three pilot studies show that there is high behavioral interest in conspecific urine, especially that of the opposite sex. These results support the accumulating evidence that the maned wolf possesses urinary reproductive semiochemicals that convey messages regarding timing of reproduction.

The 2011 study indicated that vanilla is an appropriate control substance for bioassay studies in the maned wolf. Wooden construction shims were preferred as bioassay scent vehicles over PVC pipe tubing because PVC is porous and retains scent molecules while the wooden shims are inexpensive and disposable after each use. Wooden sticks were chewed on several occasions, so I recommend attaching them to the outside of the enclosure fence. Alternatively, Chapter 4 explores the use of sand piles as an even better bioassay scent vehicle.

Overall, these pilot studies would have been expanded to include more subjects or more observation sessions except for a severe lack of urine to use as bioassay material. The urine used as bioassay material was always the limiting factor in the experiments reported here. Therefore, I strongly recommend consistent efforts for urinary sample collection for several individuals for an entire year leading up to a bioassay study. In that way, there would be enough urine for a robust bioassay experimental design and the urine would be minimally aged.

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Marieke Kester Jones graduated from Ridge High School in Basking Ridge, New Jersey in 2005. She received her Bachelor of Science degree from Tufts University in Medford, MA in 2009 where she double-majored in Biopsychology and Environmental Studies. After college graduation she worked as an intern at the Smithsonian's Conservation and Research Center, which is now the Smithsonian Conservation Biology Institute. It was there she met Dr. Nucharin Songsasen and together they conceived of this project.