Scent-Marking: Investigating chemosensory signals in wolf urine

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ABSTRACT

Identifying the best control method for problematic wildlife is an ever present issue in wildlife management. Popular control methods have ranged from lethal techniques, extirpating the animal, to multiple non-lethal methods focused on deterring undesired behavior. In the past, lethal methods were the preferred choice. However, with increased awareness of the need for biodiversity conservation, new management methods focus on non-lethal control, with emphasis on exploiting aspects of naturally occurring organismal behaviors and ecology. Over the past decade, technological advances in extraction method's and equipment have also developed new techniques providing a broader range of information about species biology for management use.

One of the most well documented conflicts between wildlife and humans is that of the wolf. Using advanced technology and new techniques, we investigated the implication of using chemosensory signals in canid urine to modify behavior as a possible non-lethal alternative in large predator management. Here we used the SBSE method coupled with improved GC/MS equipment to analyze the volatile organic compounds in the urine of four canid species, gray wolf (*Canis lupus*), red wolf (*Canis rufus*), wolf-dog hybrids (*Canis familiaris*) and the domestic dog (*Canis familiaris*) in order to create working urinary profiles. The extraction method identified several compounds also seen in the urinary profiles of other large predators. In addition, similarities and differences were also noted between taxa and the sexes, and these can be further explored in future studies.

Two identified urinary compounds, acetophenone and methyl propyl sulfide, were selected for further behavioral evaluation. We focused on these compounds and their influence as chemosensory signals triggering urine marking events in both the gray wolf and red wolf. Behavioral observations of the effects of these two chemicals indicated they elicited responses from captive wolves. At each of the three study sites, the combination of these chemicals produced urine-marking events along the territory boundary by dominant animals. As a result, the

investigation focused on what triggered the urine-marking events, the chemicals themselves, their combination, or the breakdown of the chemicals producing other odorants. It was found that there was no significant degradation of the chemicals over time and environmental conditions produced no significant breakdown of the acetophenone prior to the addition of methyl propyl sulfide. This posed a number of new questions and illustrated the need for additional behavioral studies.

The results of this study analyzing chemosensory signals in canid urine, provides biologists with new information to aid in the development of new non-lethal management strategies for handling problematic wildlife as well as providing useful information for future research involving reproduction, predator/prey dynamics, territory maintenance, and a host of other studies focusing on animal ecology in association with chemosensory signaling.

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Chapter Three: Chemical analysis of volatile organics in urine of multiple canid species

Dr. David Hosken and Dr. Sasha Dall provided guidance and assistance in the development of the experiment and the preparation of the manuscript. I selected the extraction method and collaborated with RTI International Laboratories for sample analysis. The extraction method parameters best suited for the sample sets were developed with the assistance Dr. James Raymer, RTI International Laboratories, and his associate Ms. J. Deese-Spruill, who ran the samples on RTI lab equipment and provided a report based on my specifications and the work agreement I approved. I collected urine samples for analysis and analyzed the data from the lab reports provided by RTI International and am first author on both manuscripts.

Chapter Four: Impact of select volatile organics from canid urinary profiles on scent-marking behaviors of wolves

Dr. David Hosken and Dr. Sasha Dall provided guidance and assistance in the development of the experiment and the preparation of the manuscript. I collected all behavioral data, conducted the analysis and am first author on the manuscript.

Chapter Five: Chemical air analysis of volatile organic breakdowns associated with scent-marking behaviors seen in canids

Dr. David Hosken and Dr. Sasha Dall provided guidance and assistance in the development of the experiment and the preparation of the manuscript. I

collaborated with Dr. James Raymer and his associate Ms. J. Deese-Spruill on developing a method to replicate field conditions in a laboratory environment. RTI International labs ran the samples and provided a report based on the specifications agreed upon and the work agreement I approved. I analyzed the data from the lab report provided by RTI International and am first author on the manuscript.

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The general discussion, conclusion summaries, applicable uses for results, and future prospects presented in this chapter, reflect my own interpretation of the research conducted in this study, under the guidance of Dr. David Hosken and Dr. Sasha Dall.

Chapter One

Introduction

Humans and wildlife frequently interact with one another with these encounters increasing as human populations expand into areas formerly inhabited by wildlife. These interactions create conflict, along with threats to human life and financial security (Treves & Karanth 2003), as both humans and wildlife compete for the use of habitat and natural resources. This competition has resulted in frequent use of lethal control measures, which in turn, results in wildlife extermination (Steneck 2005). However, with increased human awareness of the need for biodiversity preservation, there has been a shift in wildlife management practices from methods primarily focused on lethal control, to methods centered on the use of non-lethal control. While the use of non-lethal methods is supported for most wildlife, as illustrated by the public use of sound to deter rodent habitation and use of odors to deter lagomorphs and ungulates from garden areas, this is not necessarily the case for large predators.

Large predators compete with humans on a variety of levels, but one of the most familiar conflicts occurs over livestock. As human populations expanded globally, livestock populations also expanded, changing ecosystem dynamics and introducing new food sources for predators (Barclay 2002; Chavez & Gese 2005; Fritts *et al.* 2003; Shivik 2004). Depredation of livestock impacts livestock owners monetarily (Musiani *et al.* 2005; Shivik 2004; Sillero-Zubiri *et al.* 2004) increasing their concerns regarding predation. It also increases their support for the use of lethal controls to reduce predation rates, increase the survival of their livestock, and thus preserve their financial investment (Shivik 2004; Sillero-Zubiri *et al.* 2004). Despite support of lethal control methods for large predators by livestock owners, there is overall public support for non-lethal management techniques that maintain predator populations while simultaneously reducing livestock depredation (Shivik 2004; Sillero-Zubiri *et al.* 2004).

One of the most publicized human-predator conflicts is that between humans and wolves. Wolf preservation has largely become a battle between conservation advocates and livestock owners exacerbated by events like the hunting of wolves along the Norwegian Border in 2004 (Bazelchuk 2005) and with delisting of wolves

in northern regions of the United States in 2008 and 2009. Here, we review the basis of the human-wolf conflict in relation to livestock predation as an example of the predator problem faced by wildlife managers. We focus on current management practices used to defuse this conflict and suggest non-lethal methods, particularly those exploiting the biology of target species, which might prove useful in effective predator management.

Human-Wolf Conflict

The conflict between humans and wolves represents one of the most well documented conflicts between predators and humans. It provides a prime example that demonstrates the challenges faced in balancing stakeholder interests, in this case conservation advocates and livestock owners. As human populations expanded in areas where wolves were once the dominant predator (Musiani & Paquet 2004), natural prey resources were reduced, while the availability of livestock increased (Breck & Meier 2004; Fritts *et al.* 2003) initiating reports of livestock depredation worldwide where humans and wolves coexist. Such reports indicate that a variety of livestock species were preyed upon by wolves, including cattle and sheep in the United States (Bradley & Pletscher 2005), reindeer in Northern Scandinavia, sheep and goats in India, and horses in Mongolia (Fritts *et al.* 2003).

With increased threat of depredation by wolves, many livestock owners turned to predator control methods that provided an immediate and permanent solution to their problem, which resulted in the extermination of wolf – and other predator – populations (Barclay 2002; Breck & Meier 2004; McIntyre 1993; Musiani & Paquet 2004; Robinson 2005; Wood 1994). In addition to this conflict with livestock owners and prior to current conservation efforts, wolves were also perceived poorly by the general public who supported the killing of wolves for pelts, protection of wild ungulate populations, disease control, and out of fear of direct threats to human life (Musiani & Paquet 2004). Despite this period of extirpation, small populations of wolves managed to persist in remote locations worldwide while a few larger populations continued to thrive in the vast wilderness areas of Alaska,

Canada, and Siberia away from daily encounters with humans and livestock. Today, the extermination of the wolf is prohibited in many countries while others maintain strict management policies, often increasing tension between livestock owners and wolves.

Conservation Issues

The first wolf conservation programs were initiated globally in the 1970's leading to the reintroduction and establishment of new wild wolf populations. Additionally, there was an expansion of existing wolf ranges through the implementation of various conservation laws facilitated by increased public support for this large canid (Fritts *et al.* 1992; Fritts *et al.* 2003).

Unfortunately, not everyone views restoration or preservation of this predator favorably. Many farmers and ranchers around the world still maintain a reserved attitude towards wolves because of livestock depredation and although in many places the general public supports wolf conservation, public attitudes about wolves vary. (see Box 1 for overview of public positions) In general, global attitudes from surveys ranging from 1972-2000 demonstrate that 60% of the population support wolf conservation efforts yet attitudes demonstrate a higher negative correlation when associated with older generations, rural inhabitants, farmers/ranchers but higher positive correlation with education and income (Williams et.al. 2002) Although general public opinion may favor wolf conservation, many farmers, ranchers, and other livestock agencies express great concern over the return of this predator and the impact it will have on livestock (Bangs & Shivik 2001; Bradley & Pletscher 2005; Breck & Meier 2004; Chavez & Gese 2005; Harper *et al.* 2005).

In an attempt to balance the priorities of both the livestock owners and conservation advocates, governments from several countries have developed compensation programs designed to reimburse livestock owners for losses attributed to wolf depredation (Fritts 1982). The downside of such programs are that wolves are often implicated in livestock loss regardless of their actual involvement. For example, in Italy, livestock owners are compensated for sheep predated by wolves, yet only 20-50% of reported depredation incidents were

confirmed wolf kills (Fritts 1982). In addition, various studies have concluded that factors such as disease, birthing problems, weather, and livestock accidents actually contribute more to livestock mortality than wolf depredation (Breck & Meier 2004). In Canada, during a four year study, only 15 of 121 livestock deaths were attributed to wolves, 4 to bears and the remaining 84% were attributed to pneumonia or consumption of poisonous plants (Fritts 1982). In the United States, during a two year study of predation rates near the Yellowstone wolf reintroduction site, only 9 of 33 cattle deaths were confirmed wolf kills and the remaining 24 were due to natural mortality (Bangs & Shivik 2001). In fact in parts of North America and Europe, wolf depredation rates per 11,000 head of livestock average less than 1% annually in most areas (Bangs & Shivik 2001; Breck & Meier 2004; Fritts *et al.* 2003; Musiani *et al.* 2005) further suggesting that the *perceived* danger of wolves to livestock is far greater than their actual impact.

Management Strategies

Management strategies for the wolf and other large predators involve lethal and non-lethal methods. Early management practices particularly emphasized lethal control and local eradication. Today, management practices for populations of predators such as the wolf focus more on the use of non-lethal methods.

Lethal Control Methods

Historically, lethal control methods included pits (Barclay 2002); steel traps (Fagerstone *et al.* 2004; Jones 2002; Musiani & Paquet 2004); snares, aerial/ground hunting (Musiani & Paquet 2004); and denning (Jones 2002) (see Box 2 for an overview of lethal control methods). In the late 1800's and early 1900's poisons such as strychnine (Fagerstone *et al.* 2004; Jones 2002); thallium sulfate and monoflouroacetic acid (compound 1080) were used as large predator management tools (Fritts *et al.* 2003). Over time, lethal control measures became more regulated, steel traps were deemed illegal in Europe and poisons such as strychnine and compound 1080 were banned in the United States, Spain, Portugal, Italy, and Greece. However both poisons are still legal and used in parts of Russia, the Middle East, and India in large predator management (Bradley & Pletscher 2005; Fritts *et al.* 2003).

Box 1. Select public positions on wolf management across the globe

Overall Support for Wolf in Surveys from 1972-2000 [Williams et al 2002]	Country / Territory	Population Dynamic	Attitude and Management Approach	References
57%	**Western United States**			
	Utah	Public Land Permitees Utah	14% Support of Reintroduction	Williams et al 2002
	Arizona	Arizona Defenders of Wildlife Members	91% Support of Reintroduction	Williams et al 2002
			5% Neutral to Reintroduction	Williams et al 2002
64%	**Eastern United States**			
	New England	New England Residents	87% Support of Reintroduction	Williams et al 2002
			3% Neutral to Reintroduction	Williams et al 2002
45%	**Alaska / Canada**			
		New Brunswick deer hunters	16% Support of Reintroduction	Williams et al 2002
43%	**Scandinavia [Norway/Sweden]**			
	Norway	Southeast Norwegian Residents	14% Support of Reintroduction	Williams et al 2002
	Norway	Norwegian Metro Areas	50% Neutral Attitude of Wolves	Williams et al 2002
	Norway	Southeastern Norway	14% Support Extirpation	Bjerke et al 1998
			37% Support Population Reduction	Bjerke et al 1998
			40% Support Population Sustained	Bjerke et al 1998
			7% Support Population Increase	Bjerke et al 1998
	Sweden	Swedish Reindeer Owners	70% Oppose Eradication and Population Protective Measures	Fritts et al 2003
	Sweden	Swedish Conservationists	91% Support Reintroduction	Williams et al 2002
			3% Neutral Attitude of Wolves	Williams et al 2002
	Sweden	Swedish Livestock Farmers	36% Neutral Attitude of Wolves	Williams et al 2002
	Sweden	Swedish non-hunters	55% Support Wolves / 31% Neutral	Ericsson et al 2003
		Swedish hunters	32% Support Wolves / 36% Neutral	Ericsson et al 2003
37%	**Western Europe and British Isles [Croatia/Scotland/Spain]**			
	Croatia	Croation Students and Foresters	50% Neutral Attitude of Wolves	Williams et al 2002
	Croatia	General Public	66% Support Sustaining Populations	Bath 2001
		Hunters	44% Support Sustaining Populations	Bath 2001
		Foresters	57% Support Sustaining Populations	Bath 2001
		Students	64% Support Sustaining Populations	Bath 2001
		General Public	45% Support Compensation for Loss of Livestock Due to Predation	Bath 2001
	Scotland	General Public [urban / rural]	43% Support of Reintroduction	Nielson et al 2007
			35% Support Reintroduction into Fenced Eco-parks	Nielson et al 2007
			14% Support Extirpation	Nielson et al 2007
		Rural	54% Concerned for Livestock	Nielson et al 2007
		Urban	35% Concerned with Attacks on Humans	Nielson et al 2007
	Spain	Livestock Owners	53% Support Extirpation	Fritts et al 2003
			38% Favor Control Methods Around Farms and Ranches	Fritts et al 2003
	Balkan Peninsula [Macedonia]			
	Macedonia	General Public	Favor Wolf Bounties Slavic legends – werewolves	Fritts et al 2003
	Central Europe [Slovakia]			
	Slovakia	General Public [local residence, students, woods people]	83% Support Large Predators such as Wolves	Wechselberger et al 2005
			78% Support Regulated Hunting Measures of Large Carnivores	Wechselberger et al 2005
			61.2% Supported Compensation for Predation of Livestock	Wechselberger et al 2005
	Central Asia [Kazakstan]			
	Kazakhstan	General Public	59% Support Extirpation	Fritts et al 2003
			3% Oppose Extirpation	Fritts et al 2003
	East Asia [Japan]			
	Japan	General Public	Moderate interest in restoration in 1996	Fritts et al 2003

Box 2. Lethal Control Methods

Method	Sub-method	Description	Cost ($)	Effectiveness	Countries	References
Pits		Deep holes dug in the ground to capture predators for extermination.	Minimal	Often traps non-target animals; trapped animals stoned to death or shot	Worldwide – Obsolete India – legal	(Fritts *et al.* 2003)
Trapping	Steel Trap	Use of steel leg traps to capture an animal it walks through an area	Moderate – High	Animals caught die of dehydration, strangulation, or shot by trapper. Occasional non-target animal capture.	Worldwide - Legal with some restrictions	(Fagerstone *et al.* 2004; Musiani & Paquet 2004)
	Snares	Wire or rope used to capture an animal as it walks through an area	Minimal	Animals caught die of strangulation, dehydration, or shot by trapper. Occasional non-target animal capture.	Worldwide – Legal with some restrictions UK – Illegal	(Fagerstone *et al.* 2004; Musiani & Paquet 2004)
Hunting	Aerial	Shooting of animals from the air	High	Animals killed are usually only those targeted.	Alaska – Legal Worldwide - Unknown	(Musiani & Paquet 2004)
	Ground	Shooting of animals from the ground	Minimal - Moderate	Animals killed are usually only those targeted.	Worldwide – Legal with some restrictions	(Musiani & Paquet 2004)
Denning		Tracking of den sites during the breeding season that result in the dens excavation and subsequently the killing of reproductive females and pups	Minimal	Animals killed are only those targeted	Worldwide – Obsolete	Jones 2002
Poisons	Strychnine	Poison used in the late 1880's to early 1900's placed in perishable fats around a decoy carcass to poison predators upon consumption	Minimal	Often kills non-target animals	Spain, Portugal, Italy, Greece, USA – Illegal Russia, Middle East, India – Legal	(Fagerstone *et al.* 2004; Jones 2002; Fritts *et al.* 2003)
	Thallium Sulfate	Colorless, odorless, tasteless poison used between 1937 and 1972 to kill predators by disrupting general cellular transport of potassium and sodium	Minimal	Often kills non-target animals	Worldwide – Unknown	(Fagerstone *et al.* 2004)
	Compound 1080 (Monoflouracetic Acid)	Developed in 1896 in Belgium and used between 1937 and 1972 in predator management. The white, tasteless substance is water soluble and is absorbed in the gastrointestinal tract, where it is metabolized to flourocitrate, impairing the Krebs cycle and resulting in death from cardiac arrest or failure of the central nervous system within 24 hours.	Minimal	Often kills non-target animals	Europe – Illegal USA – Illegal Australia – Legal as it occurs naturally in many native plants New Zealand – Legal as wildlife management tool	(Fagerstone *et al.* 2004; Fritts *et al.* 2003)

Non-lethal Control Methods

The use of non-lethal management techniques has only recently been utilized in predator management. Non-lethal methods can be divided into two general categories, aversive stimuli and disruptive stimuli (see Box 3 for an overview of non-lethal control methods). Aversive stimuli are defined as "stimuli that cause discomfort, pain, or an otherwise negative experience and are paired with specific behaviors to achieve conditioning against these behaviors" (Bangs & Shivik 2001). Also known as secondary repellents, these stimuli include conditional flavor avoidance and electric shock through a neck collar. Conditional Flavor Avoidance (CFA) uses lithium chloride as an aversive conditioning technique to deter prey consumption, but not predatory behavior (Bangs & Shivik 2001; Fritts 1982; Fritts *et al.* 2003; Mason *et al.* 2001; Shivik 2004). Research on electric shock from dog training collars, which emit an electrical charge through the animal when it makes contact with a boundary wire, is currently being expanded to determine its effects on wolf depredation (Bangs & Shivik 2001; Breck & Meier 2004; Musiani *et al.* 2005; Shivik *et al.* 2003; Shivik 2004) with one study in 2005 showing reduction in predation when in conjunction with human monitoring in a command post (Shultz *et al.* 2005). Adverse stimuli offer an alternative to lethal control methods, however, they are often difficult and in the case of the electric shock collars expensive to apply in management situations (Shivik *et al.* 2003). The expense of implementing electric shock collars poses a significant drawback to economically challenged countries. Other problems imposed by adverse stimuli include the inability to control which animals come in contact with CFA, and the capture and physical collaring of animals for effective electric shock methods.

Disruptive stimuli are defined as "undesirable stimuli that prevent or alter particular behaviors of animals" (Bangs & Shivik 2001). Also called a primary repellent, these stimuli are designed to interrupt predator hunting patterns (Shivik *et al.* 2003) and can involve chemical, visual or auditory stimuli (Breck *et al.* 2002; Mason *et al.* 2001; Shivik *et al.* 2003). Natural canid behavior includes being inherently "wary", which makes these animals particularly susceptible to non-lethal disruptive stimuli (Shivik *et al.* 2003). There are several disruptive stimuli being used by livestock

owners, either individually or in combination, and these include chemical repellents, electric fences, livestock guardian dogs, and scare devices (Box 3).

Chemical repellents utilize chemical agents to induce sickness through ingestion, irritation or fear, and include chemicals such as capsaicin, mustard oil and ammonia (Mason et al. 2001), while electric fences (Bangs & Shivik 2001; Breck & Meier 2004; Musiani *et al.* 2005; Shivik 2004) provide an electrical shock when predators make contact. The effectiveness of these primary repellents is somewhat limited due to predator habituation. Guardian animals (Bangs & Shivik 2001; Musiani *et al.* 2005) such as the domestic dog have a reported encounter and predator deterrent success rate from 66-90% , making them an effective tool in the reduction of livestock depredation in Europe, Asia, and in the United States (Coppinger & Coppinger 1992; Fritts *et al.* 2003; Smith *et al.* 2000). However, reports of livestock guardian animals killed by wolves are on record in North America and Europe, raising the question of guardian animal safety (Coppinger & Coppinger 1992). Scare devices include riot control ammunition, cracker shells, and flagging (Bangs & Shivik 2001; Fritts *et al.* 2003; Musiani & Paquet 2004; Shivik *et al.* 2003; Shivik 2004). In addition, other devices such as pyrotechnics, strobes, and sirens are used to frighten or scare predators (Bangs & Shivik 2001; Breck *et al.* 2002; Breck & Meier 2004; Fritts *et al.* 2003; Mason *et al.* 2001; Shivik 2001; Shivik *et al.* 2003; Shivik 2004). Between 1978-1986, such devices were used in the United States with questionable efficiency as two incidents of wolf depredation were reported within 30-45 meters of strobes and flashing lights (Fritts *et al.* 1992). Other canid predators have also been documented traveling between the lights and resuming livestock predation (Fritts 1982). Despite many inefficient scare devices, one promising instrument known as the radio activated guard [RAG] is being tested to keep radio-collared animals out of small livestock areas (Breck *et al.* 2002; Mason *et al.* 2001; Shivik 2001; Shivik *et al.* 2003). The device is activated when the radio collar of the wolf is detected by RAG, which then sets off strobes, lights and sirens designed to disorient the predator. The device contains 30 different recorded sounds and is designed to broadcast a different sound at subsequent triggering in an attempt to prevent habituation (Shivik 2001; Shivik *et al.* 2003).

Box 3. Non-lethal Control Methods

Method	Sub-method	Description	Cost ($)	Effectiveness	Countries	References
Conditional Flavor Avoidance [CFA]		Non-lethal chemicals (lithium chloride) give predator's negative experience when consuming treated meats; primary focus- deter future consumption of same food.	Minimal - Moderate	Deters consumption but not predatory behavior. Inability to control contact by non-target animals.	Worldwide Uses	(Bangs & Shivik 2001; Fritts 1982; Fritts *et al.* 2003; Mason *et al.* 2001)
Electric Shock Collars		Collars utilize electrical currents triggered when predator is within range of radio frequency emitted by ground wire	High $200 - $300 per animal	Predators become habituated and collaring animals is costly Effectiveness – 1-9 months on coyotes; ineffective on wolves	Worldwide – minimal use; Banned in Wales in June 2008	(Bangs & Shivik 2001; Shivik *et al.* 2003; Shivik 2004; Shivik 2006)
Chemical Repellents	Capsaicin and Capsicum Oleo Resin	Active ingredient in 'hot sauce' making the animal sick or causing irritation to eyes, ears, nose and throat surfaces	Minimal – Moderate	Predator habituation and inability to control non-target animal contact with chemical	Worldwide Uses	(Mason *et al.* 2001)
	Allyl Isothiocyanate	Active ingredient in mustard oil and ammonia inducing sickness, irritation, or fear in an animal	Minimal – Moderate	Predator habituation and inability to control non-target animal contact with chemical	Worldwide Uses	(Mason *et al.* 2001)
	Quebracho	Astringent tannins that induce vomiting in animals or cause severe physical irritation	Minimal - Moderate	Predator habituation and inability to control non-target animal contact with chemical	Worldwide Uses	(Mason *et al.* 2001)
Electric Fences		Electrical currents running in fences designed to produce a voltage shock when animal makes contact	Moderate – High	Predators become habituated rendering method ineffective.	Worldwide Uses	(Bangs & Shivik 2001; Breck & Meier 2004; Musiani *et al.* 2005; Shivik 2004)
Guardian Animals		Animals such as domestic dogs, donkeys, llamas, etc. used to deter predation based on protective nature established toward livestock. Animals will engage a predator or simply deter predation based on size and shape.	Moderate – High $200-$450 initial cost per animal $250 maintenance cost per year per animal	Predators become habituated; no longer deterred by size and shape. Guardian animals often killed engaging predator. Proven quite effective in conjunction with other non-lethal methods.	Worldwide Uses – United States, Europe, and Asia	(Bangs & Shivik 2001; Coppinger & Coppinger 1992; Fritts *et al.* 2003; Musiani *et al.* 2005; Shivik 2006; Smith *et al.* 2000)
Scare Devices	Riot Control Ammunition	Riot control ammunition consists of non-lethal 12-guage bean bag shells designed to disorient and scare predators.	Minimal	Predators become habituated	Worldwide Uses	(Bangs & Shivik 2001; Shivik 2004)
	Cracker Shells	Exploding noise makers designed to harass predators	Minimal	Predators become habituated	Worldwide Uses	(Bangs & Shivik 2001; Fritts *et al.* 2003)
	Flagging	Technique developed in Eastern Europe, also known as fladry, utilizing strips of flagging placed along fences and trees that moves sporadically frightening predators	Moderate – High $781 per km fladry $1328 per km turbo fladry	Predators become habituated Effectiveness – 60 days wolves; >2 days coyotes; ineffective on black bears	Worldwide Uses	(Bangs & Shivik 2001; Fritts 1982; Fritts *et al.* 2003; Musiani & Paquet 2004; Shivik *et al.* 2003; Shivik 2004; Shivik 2006)
	Pyrotechnics, strobes, and sirens	Designed to spontaneously disorient and scare the predator	Minimal – Moderate $50 - $200 per unit	Predators become habituated Effectiveness – wolves (several days); coyotes (several days)	Worldwide Uses	(Bangs & Shivik 2001; Breck *et al.* 2002; Breck & Meier 2004; Fritts *et al.* 2003; Mason *et al.* 2001; Shivik 2001; Shivik *et al.* 2003; Shivik 2004; Shivik 2006)
	Radio Activated Guard [RAG]	Radio Active Guard -to keep radio-collared animals out of small livestock areas. Picks up the radio frequency of collared wolves and setting off strobe lights and sirens.	High $3000 per unit	Predators become habituated Effectiveness – wolves (3 months); black bear (weeks)	United States – minimal use	(Breck *et al.* 2002; Mason *et al.* 2001; Shivik 2001; Shivik *et al.* 2003; Shivik 2006)
Species Biology	Relocation	Transportation of animals from original home range to new locations in order to deter unwanted behavior	Moderate – High	Inability to prevent future predation at relocation site; expense and time to find suitable location; inability to maintain healthy reproductive health of relocated animal	Worldwide Uses	(Bangs & Shivik 2001; Breck & Meier 2004; Fritts *et al.* 2003; Musiani *et al.* 2005)
	Fertility Control	Contraceptive drugs /devices to prevent pregnancies in animals; delivered orally, topically, via injection, or surgically implanted	Moderate – High $600 per animal	Method is not 100% effective Lasts approximately 2-3 years [coyotes]	Worldwide Uses	(Fritts *et al.* 2003; Musiani & Paquet 2004; Shivik 2006)
	Behavioral modification using olfactory receptors	Use of biological aspects of a species to regulate or modify behavior [i.e. scent-marking]	Unknown	New technique currently under investigation	N/A	

These previous techniques all rely on introducing foreign chemicals and artificial stimuli such as strobes and lights to deter predators, but a more focused approach may be to target aspects of wolf ecology. Several related studies to this approach have been investigated to include relocation and birth control. Through the use of species biology, the understanding of wolf territoriality and wolf behaviors, relocation has been a non-lethal technique used extensively for wolves persistent in livestock depredation (Bangs & Shivik 2001; Breck & Meier 2004; Fritts *et al.* 2003; Musiani *et al.* 2005). However, relocation of wolves to new territories is often time consuming as wildlife managers try to find vacant locations for release of particularly problematic individuals (Breck & Meier 2004). Exploiting species biology underpinned by studies conducted on wolf reproduction have led to the development of fertility control methods to help reduce wolf numbers in areas of high predator populations, especially those around livestock (Fritts et al. 2003; Musiani & Paquet 2004). Despite successful results in captivity, employment of fertility control methods in wild populations is extremely difficult and at times ineffective. Another avenue of species biology that has been partially investigated involves wolf scent-marking behavior. Scent-marking itself is an important aspect of olfactory communication in most mammals including canids (Brown & Johnston 1982; Ralls 1971; Zub et al. 2003), and consist of leaving marks with skin glands, urine, and feces on objects such as tree trunks, small shrubs, and rocks (Bowen & Cowan 1980; Nunez & Miguel 2004; Peters & Mech 1975; Ralls 1971; Regnier & Goodwin 1977; Sillero-Zubiri & MacDonald 1998; Vila *et al.* 1994). It has been determined that most vertebrate scent marks contain unique sets of constituents broadcasting specific signals recognized by other vertebrate species (Regnier & Goodwin 1977; Wyatt 2003). When other animals detect these chemical indicators in the environment, they modify their behavior based on the constituents with which they interact (Yahr & Commins 1983). Wolves, for example, scent-mark as a social queue indicating dominant status to both the pack and neighboring wolves (Asa *et al.* 1990) and also to mark external territory boundaries and to create internal-territory orientation features. It has been calculated that internal-territory marking allows wolves to encounter an olfactory signal around every two minutes and a urine scent-mark every three minutes (Bowen & Cowan 1980; Brown &

Johnston 1982), suggesting that canids scent-mark not only to establish borders, but to orient themselves within their own territory (Nunez & Miguel 2004; Paquet 1991. It has been suggested that foreign scent marks incite an avoidance response in wolves (Briscoe *et al.* 2002; Paquet 1991; Peters & Mech 1975) and may link unfamiliar territory to behavioral aversion (Briscoe *et al.* 2002). In turn, this deters "trespassing" into neighboring territories, and when wolves encounter a foreign scent their rate of scent-marking increases (Bowen & Cowan 1980; Paquet 1991; Peters & Mech 1975; Ralls 1971; Zub *et al.* 2003). Pack movement and scent-marking resulting from foreign scents can lead to territorial patterns that develop a packs home range because most wolves remain near existing scent marks and increase marking behavior in the presence of familiar scents (Briscoe *et al.* 2002). Support for this is evident in dispersing or foreign wolves demonstrating a reluctance to mark unfamiliar areas or areas with high volume marking by other packs. Because scent marking plays such an integral role in wolf ecology and seems to be a key means of delineating and determining movement and movement patterns, this seems like one avenue for controlling wolf-livestock interactions that should be subject to further investigation. If managers could exploit aversive behavior to foreign scent, managed scent marking could prove to be a tremendous management tool.

Conclusion

Many of the issues identified within the basis of the human-wolf conflict also apply to human interactions with other large predators and are characteristic of wildlife conflicts in general. Despite the current general focus on biodiversity preservation, negative attitudes harbored by agricultural communities, perhaps due to social or economic pressures, are not conducive to successful wolf or other large predator conservation (Musiani & Paquet 2004). This review highlights a variety of predator management strategies and reveals a need to find and test new methods that could be instrumental in resolving predator conflict issues, while also minimizing livestock loss (Bradley & Pletscher 2005). We feel that techniques exploiting predator ecology may be particularly helpful in this regard, and for wolves, scent-marking is likely to prove especially fruitful. The development of effective anti-

predation tools would clearly have a positive effect on wolf conservation, and if we can develop effective techniques centered around methods designed to deter depredation of livestock using non-lethal methods, we impact not only human relations with predators, but aid biodiversity conservation as whole.

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Chapter Two

Analysis of Volatile Organics in Canid Urine Using Stir Bar Sorptive Extraction [SBSE]

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Abstract

Advancements in analytical methods in the past decade have produced more effective extraction methods as well as instrumentation. These advancements such as the SBSE method allow for reliable analysis of small quantity and dilute sample sets. The processing of dilute samples becomes important when working with ecologically relevant material such as urine collected from snow. The SBSE method was used to analyse dilute urine samples of both the grey wolf (*Canis lupus*) and the domestic dog (*Canis lupus familiaris*) producing ninety five identified organic compounds with thirty four being shared between the two species. Individual organic compound urinary profiles were developed for each taxon with 31 organic compounds associated only with the grey wolf and 30 associated only with the domestic dog. Additionally, the SBSE method identified several organic compounds seen in urinary profiles run in the early eighties from bladder extracted urine, including 2-pentanone, furfural, 4-heptanone, 2-heptanone, benzaldehyde, acetophenone, and nonanal, thus verifying the approaches veracity.

Key words: *Canis lupus*, *Canis lupus familiaris*, chemical signals, stir bar sorptive extraction, urine

Introduction

Analytical methods used to identify organic compounds in biological samples play a vital role in unlocking the mysteries of semio-chemical signals in animal communication from insects (Moore, 1997; Sharma et al., 2012; Ingleby, et al., 2013) to mammals (Wyatt, 2003; Soini et al., 2005; Zhang et al., 2005). For years studies have been conducted on a variety of species ranging from insects to mammals in order to identify organic compounds responsible for triggering specific behaviors. One of the most useful biological samples analyzed for these signals is urine. Urine contains a plethora of organic compounds that signal such things as gender (Anderson & Vulpius 1999; Raymer, Weisler et al., 1984; Zhang et al., 2005), reproductive status (Anisko, 1976; Asa, et al., 1990; Packard, 2003), and territory occupancy (Peters and Mech 1975; Paquet 1991; Wyatt, 2003; Zub, et al., 2003). As the need to understand these biological signals expands, new analytical technologies and sample preparation techniques are developing to meet the growing demands.

In the last decade alone, significant advances have been made in both the methodologies and technologies used to analyze bio-chemicals (Mitra et al., 2003; David et al., 2007; Kole et al., 2010). These result in an improvement in compound separation and detection, while also providing an overall fast and cost effective means of bio analysis (Kole et al., 2010). For example, current gas chromatograph (GC) instruments have become so effective in the separation of compounds within a sample that they can theoretically separate over 300 solutes in a single run (David et al., 2007), while the increased sensitivity of the mass spectrometer improves detection and identification (David et al., 2007; Laaks et al., 2012). When combined, these developments provide an effective tool for the analysis of a variety of samples and are a preferred tool for most researchers working with aqueous biological matrices (Tienpont et al., 2002) such as urine.

Yet despite recent technical advances, it is often the sample preparation or extraction technique that most impacts bio-analysis (Mitra et al., 2003; David et al., 2007; Nerin et al., 2009). It is this step in the analytical process that accounts for

up to 80% of the time invested in sample analysis (Kole et al., 2010), making it the most labor intensive and error prone aspect of the procedure (Kole et al., 2010). This significantly impacts sample throughput and dictates cost (Tienpont et al., 2002). Therefore, when identifying an extraction technique, it is important to look at the sample and determine what information is desired. Despite the number of extraction techniques available, it is currently not possible to use only one and glean all the potential chemical information from a sample (Mitra, 2003; Soini, et al., 2005). Therefore, the nature of the sample to be analyzed will dictate the extraction method most suitable, and as a result, selecting the appropriate sample preparation and extraction method is paramount in ensuring not only a cost effective analysis, but also one that provides an accurate and reliable assessment of the organic compounds within a sample (Urbanowicz et al., 2011).

However, at least one concern remains for field biologists seeking cost effective analyses, and this lies in the collection of field samples deposited by animals, as often times the acquired samples are collected in small quantities or the samples may be dilute as with snow collections of urine. Samples such as these can prove problematic to certain analytical extraction methods that require large volumes of sample as well as repeated extractions for sufficient analysis (Laaks et al., 2012).

One extraction technique proven to successfully determine low traces of organic compounds in aqueous matrices is the stir-bar sorptive extraction (SBSE) method (David et al., 2003), making it a cost effective and ideal method for analyzing snow and dilute urine samples. The SBSE method was developed by the Research Institute of Chromatography (Kortrijk, Belgium) (Baltussen et al., 1999; Sanchez-Rojas et al., 2009; Lancas et al., 2009) and was commercialized under the name “Twister” by Gerstel (Mulheim, Germany) (Sanchez-Rojas et al., 2009). The SBSE method has been used successfully in identifying compounds in small or dilute sample sets for a variety of applications from the identification of organic pollutants in water samples (Tienpont et al., 2002; David et al., 2003), to the profiling of flavors in food (Tienpont et al., 2002; David et al., 2003) to multiple biological studies ranging from aquatic organisms, to insects, to mammals, including many

mammal studies involving the analysis of various urine compounds (Soini et al., 2005; Zhang et al., 2005; Novotny et al., 2007).

The current study was designed to apply the SBSE method coupled with GC/MS to analyze the organic compounds in dilute samples of canid urine and to use current computerized approaches to data reduction and analysis. Here we develop the urinary profile of the wolf (*Canis lupus*) and look at possible implications for the use of a urinary profile produced by the SBSE method in wildlife management.

Materials and methods

Sample Collection and Preparation

Urine samples were obtained from various North American captive facilities and private owners. Collection methods consisted of the acquisition of urine from snow samples (DelGiudice, et al.,1989; Darnell, et al., 2005; Christianson & Creel, 2010). Snow samples were collected using a sterile vial to scoop up yellow snow by staff and personnel at respective facilities. All samples were stored in a -20°C freezer prior to preparation. Samples obtained in this study were collected in both the spring and winter of 2008 and included 15 gray wolf male samples from five different male gray wolves. Individual samples from each sample set (gray wolf male were then pooled prior to analysis in order to alleviate organic variations among individuals as a result of diet or other environmental conditions (Raymer J. H., 1984). Pooling of the samples consisted of combining 2ml of sample from each sample collected for individuals and placing it into a 50ml polyethylene vial to create a composited, mixed sample for each taxon. Mixed samples were then analyzed using the standard Stir Bar Sorptive Extraction [SBSE] Method as follows.

Sample Analysis

The SBSE method employs the use of a magnetic stir-bar or twister bar that reduces manual handling and preparation errors typically associated with sample preparation. The stir bar consists of a magnetic rod with a glass jacket that has been coated with a polymer, Polydimethylsiloxane (PDMS), (David et al., 2003;

Soni et al., 2005; Kole et al., 2010) and the bar is placed directly into the sample for the extraction phase which takes place during the stirring process (Baltussen et al., 1999). The PDMS coating is a sorbent material and is therefore non-porous preventing organic compounds within the sample from bonding to the polymer on the stir bar but rather allows the compounds to remain in dissolution (Baltusen et al., 2002). The PDMS sorbent, therefore, is not affected by high water content in dilute samples since all compounds have their own partitioning or sorption rate with the polymer (Baltussen et al., 1999; Lancas et al., 2009). Once the extraction is complete, the stir bar is removed from the sample and placed in a thermo desorption system where the organic compounds are then vaporized and sent directly into the GC/MS for analysis (Baltussen et al., 1999).

Conditioning and Screening of Material

Desorption tubes were comprised of non-salinized glass [1/4" x 3"], with a glass wool plug [1cm]. The tubes were conditioned on the Perkin Elmer Turbomatrix Automated Thermal Desorber [ATD] by heating to 275°C for 60 minutes with a flow of 25mL/min He. The flow was diverted from the splitter lines to prevent contact with the cold trap on the ATD. Polydimethylsiloxane [PDMS] coated stir bars (10mm, 0.5mm film thickness, 24µl PDMS volume, Gerstel GmbH, Mülheim van der Ruhr, Germany) were inserted into conditioned tubes and both were analyzed for contamination by desorption at 260°C on the ATD with a flow rate of 25mL/min and trapping on Tenax at -30°C. The trapped analytes were then introduced to the GC by heating the cold trap at 40°C/sec to 350°C and swept to the head of the column at 19mL/min via a heated transfer line (225°C). The compound separation and characterization was performed using an Agilent 6890 GC with a RTX-5ms column [30m x .320mm ID x .5um film, Restek] and an Agilent 5973N mass-selective detector, operated in full scan mode (30-550amu) in electron impact (EI) mode where the detector was a continuous dynode electron multiplier, and ChemStation [version E.02.00.493] data system. Samples were analyzed only after the system was shown to have minimal background.

Standard Samples

A standard solution containing hexanol, benzaldehyde, octanol, and decanoic acid prepared at 20 ng/mL each analyte in water [spiked as a methanol solution into water] was subjected to stir bar extraction and analyzed in the same manner as the canid urine samples. Analysis of this sample provided assurance that chromatographic separation performance and MS sensitivity were adequate to insure the representative chemical class presentation at consistent sensitivity.

Canid Urine Sample Extraction

Samples were removed from -20°C freezer and thawed completely at room temperature. Polydimethylsiloxane [PDMS] stir bars were added to 4500ul of diluted sample and vortexed at room temperature for 1 hour. The stir bars were then placed into conditioned desorption tubes and desorbed for 15 minutes on the automated thermal desorber [ATD]. The effluent was trapped at -30°C on a secondary Tenax trap. The secondary trap was desorbed at 350°C for 15 minutes. Trapped analytes were introduced to the gas chromatograph [GC] by heating the cold trap at 40°C/sec to 350°C and swept into the head of the column [Restex RTX-5ms, 30 m X 320um X 0.5 um] at 17ml/min via heated transfer line [225°C]. GC parameters are detailed in Table 1.

Table 1. GC / MS Operating Parameters

Parameter	Setting
Oven	
Initial Temperature	40°C
Initial Time	5 min
Rate	3°C min $^{-1}$
Final Temperature	200°C
Final Time	10 min
Total Run Time	68.33 min
Injector – External device [ATD] Column	
Head Pressure	17 psi
Flow	3.0 mL min $^{-1}$
Mass Spectrometer	
Transfer Line Temperature	250°C
Mass Scan Range [full scan]	30-550 amu
Electron Impact [EI]	70 eV
Scan Rate	1.44 scan/sec.

Following data acquisition, data files were processed for peak deconvolution and ion/retention time alignment using the following software packages: Automated Mass spectral Deconvolution and Identification Software [AMDIS]/ NIST, and METabolomics Ionbased Data Extraction Algorithm [MET-IDEA] (Chan, et al., 2011). AMDIS deconvolluted the mass spectral data to differentiate very closely eluting components and created a list of ion/retention time values. MET-IDEA used the ion/retention time list to align the ions and retention times and to calculate normalized compound areas which allowed for comparison among all the samples. NIST library spectral matches were used to identify tentative chemical compounds in the urinary profile. The non-authenticated compounds were then used to develop a tentative urinary profile for the gray wolf.

Results

Materials and Standards

A conditioned desorption tube blank and a stir bar blank were analyzed along with the check standards and urine samples. The desorption tube or system blank was essentially free of peaks. The stir bar blank contained siloxanes typically thought to be associated with the glass wool plugs or glass tubes and phthalates thought to be associated with the o-rings used in the ATD along with two fatty acids, hexadecanoic acid and octadecenoic acid respectively and were present at the approximate retention time regions of 53 minutes and 60 minutes.

A check standard was conducted in between urine samples and bracketed urine samples and system blanks with the purpose of monitoring instrument response during the course of the procedure. Results are presented in Table 2 and demonstrate method/instrument reproducibility/stability.

TABLE 2. Check Standard Area of Response

		Ck Standard @ 20 ng/ml	Ck Standard @ 20 ng/ml		
Compound	Retention Time	Area Response	Area Response	% Difference	%RSD
Hexanol	6.394	425308	438412	3.08	2.15
Benzaldehyde	10.315	301317	261681	-13.15	9.96
Octanol	16.326	1280401	1289407	0.70	0.50
Decanoic acid	31.058	10346001	10767198	4.07	2.82

An overlay of urine samples, the system blank and the stir bar blank Figure 1 demonstrates that instrumentation and the adsorption materials do not contribute significantly to the urinary profiles.

FIGURE 1. Comparison of wolf urine sample, system blank, and stir bar blank

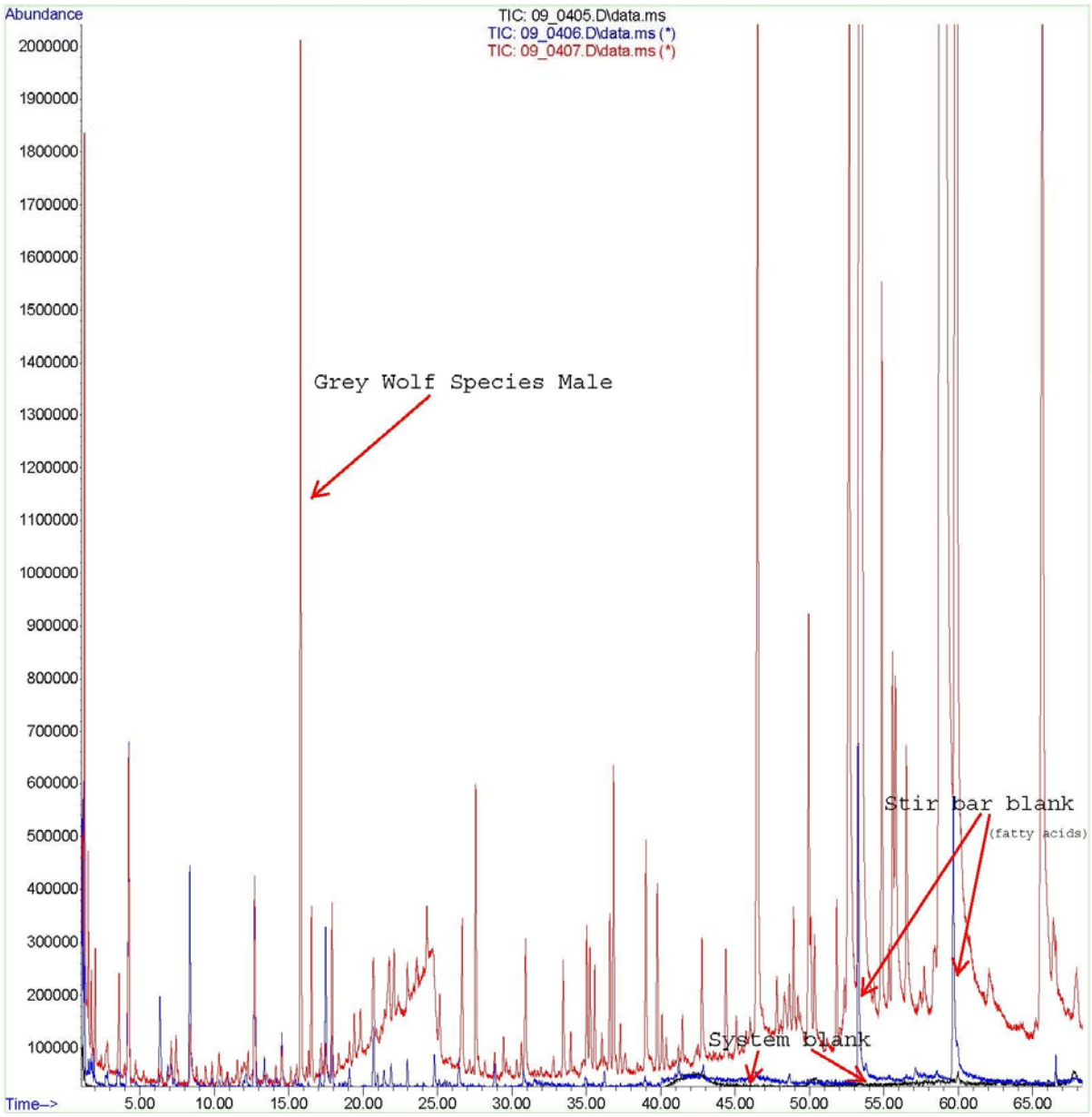

Figure 1. Comparison sample of wolf urine, system blank, and stir bar illustrating that system instrumentation and adsorption materials did not significantly contribute to urinary profiles.

Canid Urine Samples

Compounds were identified using NIST library spectral matches from which a tentative target list of compounds was developed for the male gray wolf male (Appendix I). All the identified compounds have a NIST spectral match of 50% or greater. Several instances of peaks with the same characteristic ion and retention times were matched across samples. The compounds included, but were not limited to, ketones and fatty acids. Most background compounds such as siloxanes and phthalates were eliminated from the analyte list because of their 100% ions (base peaks) were listed as background ions in a MET-IDEA exclusion list.

In addition, the fronting peak that elutes in the grey wolf (Figure 2) in the retention time region of 18 to 25 minutes was identified as urea. This peak distorts the profile, but does not prevent data interpretation.

FIGURE 2. Reconstructed ion chromatograms from GC/MS analysis of volatile compounds found in the urine of male gray wolf.

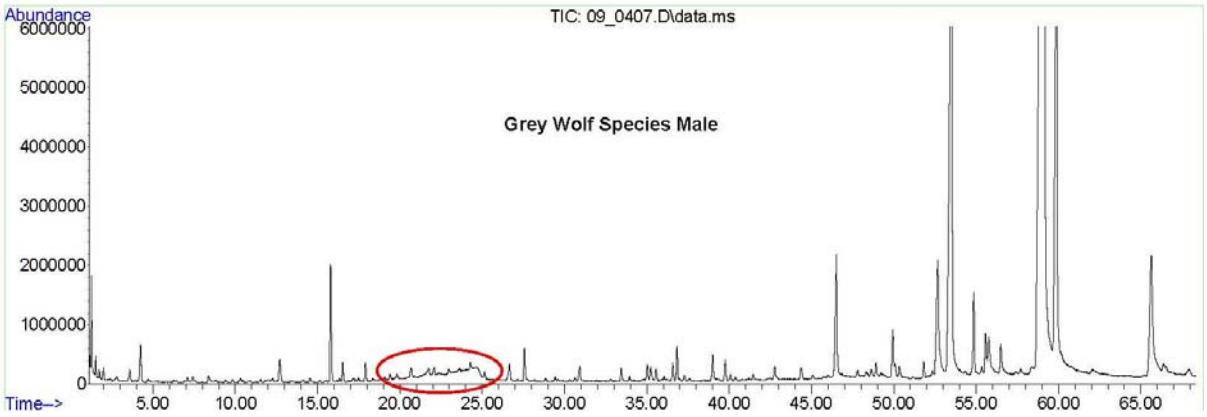

Figure 2. The reconstructed ion chromatogram from GC/MS analysis of grey wolf urine shows a fronting peak in the retention time region of 18-25minutes which was identified as urea. The peak distorts the profile but does not prevent data interpretation. The observation that urinary components are reduced in the entire set of urine samples suggests that marginally diluting urine samples does not decrease the amount of organics seen in the urinary profile.

Discussion

Overall, the extraction technique and data analysis approach of the SBSE method coupled with GC/MS, identified sixty six organic compounds. Within the non-authenticated chemical profile, the SBSE method also found several significant chemicals known to impact scent-marking in carnivores from previous studies: benzaldehyde, 2-heptanone, nonanal, and acetophenone. (Jorgenson, et al., 1978; Raymer, 1984; Andersen & Vulpius, 1999; Burger, et al., 2008). In addition, the fronting peak identified as urea in the diluted wolf - urine sample suggests that even though urinary components are reduced, marginally diluting urine samples do not decrease the quantity of organics seen in the urinary profile. Recently a protocol was developed for global urinary metabolic profiling using GC/MS including the use of urease to minimize the contribution of urea to metabolite profiles (Chan et al, 2011). Despite not using the protocol here, our results still indicate that this extraction method is reliable when working with small quantity or dilute sample sets.

If we compare the SBSE urinary profiles to previously published gray wolf and domestic dog chemical urine profiles from the early eighties, there is considerable overlap, as expected, especially for organic compounds like 2-pentanone, furfural, 4-heptanone, 2-heptanone, benzaldehyde, acetophenone, nonanal, methyl butyl sulfide and methyl propyl sulfide (Raymer J. H., 1984).

There are two possible explanations for the overall differences between the chemical urinary profiles of the gray wolf conducted in the early eighties and the current study. Firstly, different analytical methods and sample preparation procedures will influence compound recovery from biological samples (Mitra, 2003; Soini, et al., 2005; Zhang, et al., 2005). Biological matrices include a variety of chemical compounds ranging from alcohols, to aldehydes, carbohydrates, esters, fatty acids, ketones, and phenols where the detection of each depends on the compounds chemical properties, making it difficult to find a method that is optimal for detecting them all (Andersen & Vulpius, 1999). Secondly the collection of the samples themselves could generate between study differences. The samples in

the current study were obtained from dilute sample sets such as urine collected from snow, whereas, samples for the previous study extracted directly from the animals bladder. These differences and the impact of degradation due to external environmental conditions on dilute samples are likely to significantly impact the organic compounds that can be detected.

In addition, samples in this study were pooled similar to the 1984 study by Raymer. However, it is important to note that running all samples independently would have allowed for statistical comparisons to further analyze the effectiveness of the SBSE methodology in identifying organic compounds for the urinary profile.

In utilizing the SBSE method to develop reliable urinary profiles of organic compounds from dilute samples of canid urine, we find that non-invasive collection methods, such as snow collection of urine, can still be used as a viable means of ecological analysis. In addition, with the tentative identification of the chemicals in urine samples from the gray wolf, the chemicals can be further tested to determine olfactory impact on behavior. Studies such as this on canid behavior are currently being conducted to aid in understanding possible chemosensory signaling in the urine of several canid species.

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Chapter Three

Chemical Analysis of Volatile Organics in Urine of Multiple Canid Species

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Abstract

Stir-bar sorptive extraction methods coupled with gas chromatography-mass spectrometry were used to analyse volatile compounds in the urine of male and female grey wolves (*Canis lupus*), male and female red wolves (*Canis rufus*), male wolf-dog hybrid (*Canis lupus familiaris*), and male domestic dog (*Canis lupus familiaris*). One hundred and forty-four volatile compounds were identified for the male canids and one hundred and two volatile compounds were identified for the female canids. Similarities and differences between taxa and sexes were documented. Species specific compounds included thirteen compounds associated only with male grey wolves, eighteen with male red wolves, seventeen with the wolf-dog hybrid, twenty-four with the domestic dog, twenty-one with female grey wolves and twenty-five compounds associated only with female red wolves. Additionally, the analysis of both the red wolf and wolf-dog hybrid urines provide chemical profiles for two canids that have not been analysed in previous studies.

Key words: *Canis lupus*, *Canis rufus*, *Canis familiaris*, chemical signals, stir bar sorptive extraction, scent-marking

Introduction

Many studies have shown that mammalian carnivores use urine as a means of communication. This includes wolverines (Wood, et al., 2009), ferrets (Zhang, et al., 2005), tigers (Burger, et al., 2008), lions (Andersen & Vulpius, 1999), foxes (Jorgenson, et al., 1978), and wolves (Mech & Boitani, 2003; Peters & Mech, 1975). Urine acts as a semiochemical signal that contains a range of potential information that is disseminated from one animal to another through olfactory communication channels. These olfactory channels regulate an animal's ability to detect and perceive volatile chemical signals (Wilson & Stevenson, 2006). Chemical signals are then processed by a single specific sensory receptor, such as in those assigned to specific pheromones, or through complex chemical stimulus mixtures as found in individual odour recognition (Wilson & Stevenson, 2006). When animals detect these chemical indicators in the environment, they can modify their behaviour in response to the information conveyed (Albone, 1984; Yahr & Commins, 1983). Understanding the information content of these urinary signals is important to our understanding of both interspecies (allomone or kairomone) and intraspecies (pheremone) forms of olfactory communication (Doty, 2010).

Interspecific communication involves a variety of forms to include chemical signals that relay messages between individuals of different species either to benefit the producer and not the receiver (allomone) or to benefit the receiver and not the producer (kairomone) (Doty, 2010). Carnivores often use these chemical cues when hunting to detect odor trails and then track their prey. At the same time, information contained in predator urine can impact prey species , causing shifts in habitat selection, foraging strategies, and breeding patterns (Christianson & Creel, 2010; Taylor & Pekins, 1991). For example, urine from several carnivores, including wolves and foxes, supresses the feeding behavior of snowshoe hare (*Lepus americanus*), while deer urine has no effect on the hare's feeding patterns (Conover, 2007). In addition, these responses have shown to be innate in some prey species, as even hand-reared prey animals like mule deer for example avoid predator urine (Conover, 2007). Understanding these types of signals in carnivore

urine in particular may facilitate the development of tools that could be used in managing predator or prey species.

Intraspecific (pheremone) communication involves chemical signals that relay messages between individuals of the same species, and include the messages relayed in urine marking (Doty, 2010). Carnivores often urine mark to indicate social position (McLeod, et al., 1996; Mech & Boitani, 2003; Ralls, 1971), food acquisition (Nunez & Javier de Miguel, 2004), physiological and nutritional states (Darnell, et al., 2005; Delgiudice, et al., 1987), reproductive status (Anisko, 1976; Asa, et al., 1990; Packard, 2003), individual identity or gender (Andersen & Vulpius, 1999; Raymer, Weisler et al., 1984; Zhang, et al., 2005), and to indicate territory possession and maintenance (Bowen & Cowan, 1980; Paquet, 1991; Peters & Mech, 1975; Rothman & Mech, 1979; Wyatt, 2003; Zub, et al., 2003). For example, behavioural studies indicate that when presented with urine; both dogs and wolves can distinguish between their own urine, the urine of other males, and the urine of females (Brown & Johnston, 1983; Doty R. , 1986). In addition, male wolves and dogs can distinguish changes in the reproductive status of females from their urine and will alter their behaviours accordingly (Brown & Johnston, 1983).

Volatile organic chemicals responsible for semiochemical signals found in biological fluids such as urine are not typically single compounds, but rather complex mixtures making it difficult to understand which chemicals trigger certain behavioural responses (Albone, 1984; Doty, 2010; Wilson & Stevenson, 2006). Chemical cues, whether interspecies or intraspecies specific, convey information through constituent concentrations and their combinations and mixtures (Wilson & Stevenson, 2006), and it is through deciphering the structural and/or chemical properties of these cues that analytical chemistry can begin to decipher the information animals glean through olfaction.

In the family Canidae, chemical profiles of urine have been reported for the grey wolf (*Canis lupus*) (Raymer, 1984; Raymer, et al., 1984), the red fox (*Vulpes vulpes*) (Jorgenson, et al., 1978), domestic dog (*Canis familiaris*) (Raymer, 1984), and coyote (*Canis latrans*) (Raymer, 1984). The chemical profile of grey wolf urine

identified sixty four different chemical compounds, while for the domestic dog (*Canis lupus familiaris*) seventy six different compounds (Raymer, 1984). Many of these chemical profiles were conducted using standard purge and trap extractions coupled with gas chromatograph-mass spectrometry analysis. In the present study, we have used the stir-bar sorptive extraction (SBSE) method, a relatively recent and more advanced method (David, et al., 2003; Soini, et al., 2005), to analyse the urine of male and female grey wolves (*Canis lupus*), male and female red wolves (*Canis rufus*), male wolf-dog hybrid (*Canis lupus familiaris*), and the male domestic dog (*Canis lupus familiaris*). The study looks at the similarities and differences in the volatile compounds of urine between species and sexes and includes two species, the red wolf (*Canis rufus*) and the wolf-dog hybrid (*Canis lupus familiaris*), which have no chemical profiles to date.

Materials and methods

Sample Collection and Preparation

Urine samples were obtained from various North American captive facilities and private owners. Collection methods consisted of an undiluted catch method, cystocintesis, and the acquisition of urine from snow samples (Christianson & Creel, 2010; Darnell, et al., 2005; DelGiudice, et al., 1989). The undiluted catch method was used for the domestic dog in which a sterile vial was attached to a pole and placed in the urine stream during a marking event. Cystosintesis samples were received from SSP coordinator and U.S. Fish and Wildlife personnel for all red wolf samples which had been collected during individual wolf exams. Snow samples were acquired for all grey wolf and hybrid animals and consisted of using a sterile vial to scoop up yellow snow upon observation of a marking event by staff and personnel at respective facilities. All samples were catalogued and stored in a -20°C freezer prior to preparation. Samples obtained in this study included 15 gray wolf male samples from five different male gray wolves; 3 red wolf male samples each from a different male red wolf; 6 gray wolf – domestic dog hybrid male samples from three different male wolf-dog hybrids; 4 domestic dog male samples

[German shepherd breed] from one male dog; 11 gray wolf female samples from four different gray wolf females; and 3 red wolf female samples each from a different female red wolf. Individual samples from each sample set (gray wolf male, red wolf male, wolf-dog hybrid male, domestic dog male, gray wolf female, and red wolf female) were then pooled by species and gender prior to analysis in order to alleviate organic variations among individuals as a result to diet, health conditions or other environmental conditions (Raymer, 1984). Pooling of the samples consisted of combining 2ml of sample from each sample collected for each individual and placing it into a 50ml polyethylene vial to create a composited, mixed sample for each taxon. Mixed samples were then analyzed using the standard Stir Bar Sorptive Extraction [SBSE] Method (Soini, et al., 2005; Chapter 2) as follows.

Sample Extraction and Analysis

Samples were removed from -20°C freezer and thawed completely at room temperature. Polydimethylsiloxane [PDMS] sir bars (10mm, 0.5mm film thickness, 24µl PDMS volume, Gerstel GmbH, Mülheim van der Ruhr, Germany) were added to 4500ul of sample and spun on high at room temperature for 1 hour. The stir bars containing the extracted urinary chemicals were then placed into conditioned desorption tubes and subjected to thermal desorption for 15 minutes at 350°C using an automated thermal desorber [ATD, Perkin-Elmer)]. Volatile compounds contained in the effluent were trapped at -30°C on a secondary Tenax trap. Analytes were then introduced to the gas chromatograph [GC] by heating the cold trap at 40°C/sec to 350°C (15 minute hold) and the desorbed VOCs were swept into the head of the column [Restex RTX-5ms, 30 m X 320um X 0.5 um] at 17ml/min via heated transfer line [225°C] and then directed to the mass spectrometer on electron impact [EI] mode [70eV] operating in full scan (30-550amu) where the detector was a continuous dynode electron multiplier. GC parameters are detailed in Table 1.

Table 1. GC / MS Operating Parameters

Parameter	Setting
Oven	
Initial Temperature	40°C
Initial Time	5 min
Rate	3°C min $^{-1}$
Final Temperature	200°C
Final Time	10 min
Total Run Time	68.33 min
Injector – External device [ATD] Column	
Head Pressure	17 psi
Flow	3.0 mL min $^{-1}$
Mass Spectrometer	
Transfer Line Temperature	250°C
Mass Scan Range [full scan]	30-550 amu
Electron Impact [EI]	70eV
Scan Rate	1.44 scan/sec

Following data acquisition, data files were processed for peak deconvolution and ion/retention time alignment using the following software packages: Automated Mass spectral Deconvolution and Identification Software [AMDIS]/ NIST, and METabolomics Ionbased Data Extraction Algorithm [MET-IDEA] (Chan, et al., 2011). AMDIS deconvolluted the mass spectral data to differentiate very closely eluting components and created a list of ion/retention time values. MET-IDEA used the ion/retention time list to align the ions and retention times and to calculate normalized compound areas which allowed for comparison among all the samples.

Results

Male Canid Urine Samples

Tentative compounds were identified using NIST library spectral matches (Figure 1) from which a target list of compounds was developed for males of each species gray wolf; red wolf; wolf-dog hybrid; and domestic dog (Appendix I). All the identified compounds have a NIST spectral match of 50% or greater. Several instances of peaks with the same characteristic ion and retention times were

matched across samples. The compounds included, but were not limited to, ketones and fatty acids. Most background compounds such as siloxanes and phthalates were eliminated from the analyte list because of their 100% ions (base peaks) were listed as background ions in a MET-IDEA exclusion list. The non-authenticated compounds were then used to develop tentative urinary profiles for each of the canids in the study.

Further analysis of the initial compound identification shows that each species contains variations of the organic compounds associated with the canid family both similar and different. We found that male gray wolf urine contained a total of seventy identified organic compounds of the 144 compounds identified in conjunction with all four canids. Of those seventy compounds, the male gray wolf had nineteen specific compounds not detected in the other three canid species (Table 2). In addition, the male gray wolf shares four of the same compounds (n-butyl methyl sulfide; 2-nitro-1,4-benzenedicarboxamide; 3-octanone; and 17-pentatriacontene) found only between itself and the male red wolf, three compounds (1-cyclopropylpentane; butyl butyrate; and dihydroactinidiolide)

FIGURE 1. Reconstructed ion chromatograms from GC/MS analysis of volatile compounds found in the pooled samples of urine by species male gray wolf, red wolf, wolf-dog hybrid, and domestic dog.

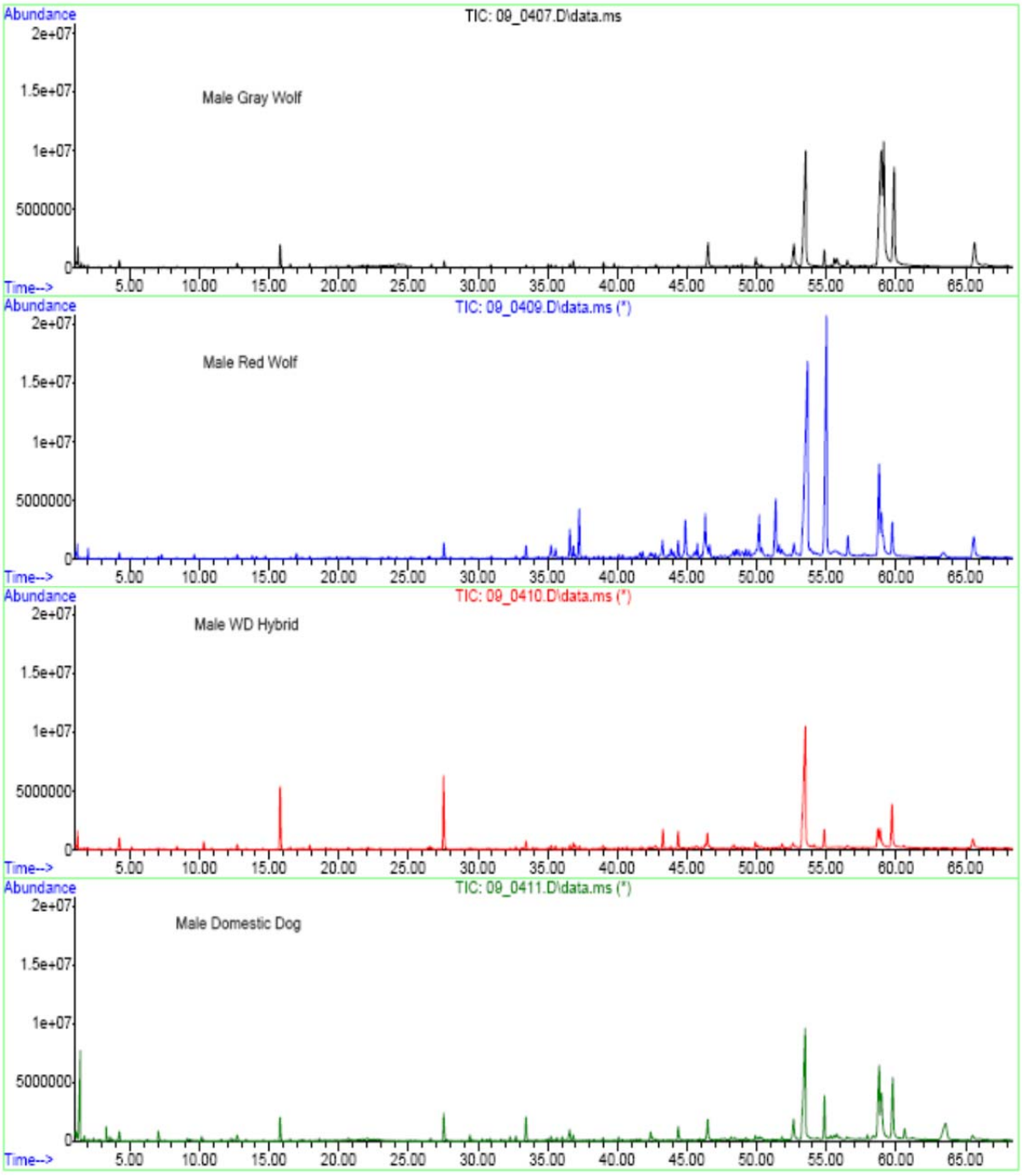

***Table 2.** Compounds in male urine specific to each of the individual male canid species

Grey Wolf	Red Wolf	Wolf-Dog Hybrid	Domestic Dog
1-butanol	3-methyl-2-hexanone	3,5-dihydroxybenzamide	propanoic acid
methyl cyclohexane	2,2,6-trimethylcyclohexanone	hexanal	methyl isobutyl ketone
diacetamide	3,5-dimethyl-2-octanone	α pinene	acetamide
n-(2-methylpropylidene) hydroxylamine	3,5-dimethyl-2-octanone	2-formyl-4,6-dimthoxy-,8,8-dimethoxyoct-2-yl benzoate	3,4,5-trimethoxy benzamide
2-methyl-2-hexanol	hexanoic acid	hexanoic acid	3-ethylcyclopentanone
1,2,3,4,5-pentamethylcyclopentene	3,4-dimethyl-2-hexanone	benzoxazole	benzonitrile
2-propylmalonic acid	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	succinaldehyde oxime	2,4,6-trimethylpyridine
imidodicarbonic diamide	dihydro-3-pentyl-2(3H)-furanone	octanoic acid	2-methyl-5-vinyl pyrazine
n-formyl-imidodicarbonic diamide	3-methyl-2-heptanone	2,3-dihydrobenzofuran	2,4,4-trimethylbut-2-enolide
2,4-decadienal, (E,E)	1,1'-diol-1,1'-bicyclopentyl	(1S-endo)(4,7,7-trimethyl-3 bicyclo [2.2.1] heptanyl) acetate	2-methyl-3-octanone
1-methylpropyl butanoate	2-(dicyclohexylphosphino)-n,n-diethylethanamine	2-undecanone	allantoic acid
dihydro-5-pentyl-2(3H)-furanone	3-ethyl,2,2-dimethyloxazolidine	bis(2,2-dimethyl propyl) disulfide	ethanedithioamide
2-propylthiazole	n-ethyl-2-methyl-5-nitrobenzeneamine	o-hexyl-hydroxylamine	2-methoxy-4-vinylphenol
3,3-dimethyl-5-phenyl-3H-pyrazole	n-(3,4-difluorophenyl)acetamide	dihydro-5-propyl-2(3H)-furanone	isobutyl isobutylrate
3-amino-2-cyclohexenone	3,3-dimethylpyrrolidine-2,5-dione	dimethyl-carbonocyanidothioic amide	5-hexyldihydro-2(3H)-furanone
polyparaben	ethyl-4-ethoxybenzoate	o-methyl S-2-diisoropylaminoethyl ethylphosphonothiolate	tridecanoic acid
E,E-2,13-octadecadien-1-ol	tetrahydro-4,6-dimethyl-2H-pyran-2-one	3',5'-dimethoxyacetophenone	1,3-dimethyl-2-imidazolidinone
1-octadecene	2,4-dimethylundecane	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-(E)-3-buten-2-one	1-nitrosopiperidine
Z-2-octadecen-1-ol	1-octadecanethiol	5-heptyldihydro-2(3H)-furanone	1-(4-hydroxy-3-methoxyphenyl)-ethanone
	tetratriacontane	lauric anhydride	4-methyl-1,6-heptadien-4-ol
	3-methylpentadecane	4-methoxy-1-methyl-bicyclo(2.2.2)octanone	dihydro-5-(2-octenyl)-(Z)-2(3H)-furanone
		octadecanoic acid	γ dodecalactone
			1,3,5-triazine-2,4,6(1H,3H,5H)-trione
			oxacyclotridecan-2-one
			oxybenzone
			Z-methyl ester-9-hexadecenoic acid
			1,2-15,16-diepoxyhexadecane

*Note: Rentention times and CAS numbers for each compound are located in APPENDIX I.

found only between itself and the male wolf-dog hybrid, one compound (undecanoic acid) between itself and the domestic dog, and six compounds (methyl propyl sulfide; methoxyphenyloxime; 2-hydroxy benzaldehyde; heptanoic acid; benzoic acid; and n-decanoic acid) shared only between itself the red wolf male and the male wolf-dog hybrid (Table 3).

We found that male red wolf urine contained a total of sixty-eight identified organic compounds of the 144 compounds identified across all four canids. Of those sixty-eight, the male red wolf had twenty-one specific compounds not detected in the other three canid species (Table 2). In addition, the male red wolf shares three compounds (2,4-diphenyl-2,3-dihydro-1,5-benzothiazepine; 2-nonanone; and decanal) found only between itself and the wolf-dog hybrid, two compounds (2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine and Isobutyl isobutyrate) found only between itself and the domestic dog, and one compound (quinoline) shared among itself, the wolf-dog hybrid, and the domestic dog (Table 2).

We found that the male wolf-dog hybrid urine contained a total of seventy identified organic compounds of the 144 compounds identified across all four canids. Of those seventy compounds, the male wolf-dog hybrid had seventeen specific compounds not detected in the other three canid species (Table 2). In addition, the male wolf-dog hybrid shares one compound (n-acetyl-2,4-difluoroaminobenzene) found only between itself and the domestic dog (Table 3). The male domestic dog urine contained a total of seventy-two identified organic compounds of the 144 compounds identified across all four canids. Of those seventy-two compounds, the male domestic dog had twenty-four specific compounds not detected in the other three canid species (Table 2).

In addition to noting the differences among each species it is also important to note that the analyte list also identified, thirty-one compounds out of the 144 compounds found in the collective male canid samples as those being found in males of all four species (Table 4).

Table 3. Organic compounds detected in male urine shared specifically between canid species

Retention Time [RT]	Compound	Grey Wolf	Red Wolf	Wolf-Dog Hybrid	Domestic Dog
3.9674	n-butyl methyl sulfide	X	x		
10.8454	2-nitro- 1,4-benzenedicarboxamide	X	x		
11.819	3-octanone	X	x		
54.3467	17-pentatriacontene	X	x		
1.9993	methyl propyl sulfide	X	x	x	
8.372	methoxyphenyloxime	X	x	x	
14.5312	salicylaldehyde	X	x	x	
21.4588	benzoic acid	x	x	x	
30.9198	n-decanoic acid	X	x	x	
16.3448	1-cyclopropylpentane	X		x	
30.6314	butyl butyrate	X		x	
37.274	dihydroactinidiolide	X		x	
17.1788	heptanoic acid	X		x	x
35.0233	undecanoic acid	X			x
9.8428	2,4-diphenyl-2,3-dihydro-1,5-benzothiazepine		x	x	
17.3115	2-nonanone		x	x	
22.9713	decanal		x	x	
3.5105	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine		x		x
30.6314	isobutyl isobutyrate		x		x
24.2749	quinoline		x	x	x
36.5715	n-acetyl-2,4-difluoroaminobenzene			x	x

Table 4. Organic compounds detected in male urine in all four canid species gray wolf, red wolf, wolf-dog hybrid, and domestic dog.

Retention Times [RT]	Compound
1.2679	acetic acid
1.7351	2-pentanone
1.9185	propanoic acid
4.6884	furfural
6.2481	4-heptanone
7.0972	2-heptanone
9.4022	4-methyl-2-heptanone
10.317	benzaldehyde
12.2782	pentanoic acid
15.7852	acetophenone
16.5213	p-tolualdehyde
17.9056	nonanal
25.132	1,3-bis(1,1-dimethylethyl)-benzene
26.6629	nonanoic acid
27.5535	2-methyl-quinoline
33.4324	2-methyl-8-quinolinol
36.0362	tetrahydro-6-pentyl-2H-pyran-2-one
36.8287	2,4-bis(1,1-dimethylethyl)-phenol
38.9953	dodecanoic acid
41.1676	benzophenone
44.362	6-heptyltetrahydro-2H-pyran-2-one
46.5078	tetradecanoic acid
49.9306	pentadecanoic acid
52.6647	Z-11-hexadecenoic acid
53.5103	n-hexadecanoic acid
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide
56.4959	heptadecanoic acid
58.9301	(Z,Z)-9,12-octadecadienoic acid
59.1043	(E)-9-octadecenoic acid
59.8669	octadecanoic acid
65.6431	ethyl (all-Z)-5,8,11,14-eicosatetraenoate

Female Canid Urine Samples

Female canid urine samples were also analyzed, however, urine samples were only obtained for the red wolf females and gray wolf females. Comparisons of the organics found in the female urine identified 102 organic chemicals (Appendix I). Of the 102 organics identified, twenty were specific to grey wolf female urine, twenty-eight were specific to the red wolf female urine (Table 5), and twenty-two were found in both the grey and red wolf female urine samples (Table 6).

Comparison of Male and Female Urine Samples: Grey Wolf (*Canis lupus*)

When looking at the organic compounds identified between sexes of specific canids we also found variations. For example between grey wolf males and grey wolf females, chromatogram data (Figure 2) details a total of 94 identified organic compounds between the two sexes with only forty-three compounds being the same in both (Table 7), twenty-three were found only in the gray wolf male and twenty-eight were only found in the gray wolf female (Table 8).

Comparison of Male and Female Urine Samples: Red Wolf (*Canis rufus*)

When looking at the organic compounds identified between genders of the red wolf species we find a total of ninety-six identified organic compounds (Appendix I) with forty-two compounds found only in the male red wolf, thirty compounds being found only in the female red wolf and twenty-three compounds being found in both genders (Table 9).

***Table 5.** Compounds in female urine specific to each of the individual female canid species

Red Wolf Female	Grey Wolf Female
1,2-diethiepane	α pinene
2,3-butanedione	γ dodecalactone
3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	1-(2,4-dimethyl-furan-3-yl)-ethanone
3-methoxy-2-butonal	1,2-benzenedicarbonitrile
6,7-dimethoxy-2H-1-benzopyran-2-one	2-nitro- 1,4-benzenedicarboxamide
3-methyl-2-heptanone	1-butanol
3,6-dihydro-3-hydroxy-6-(1-methylethoxy)-2H-pyran-2-methanol	1-nonene
3-ethylcyclopentanone	2,3-octanedione
2-methyl-3-hexanone	2,4-decadienal, (E,E)
2,4-dimethyl-3-pentanone	2-methyl-2-hexanol
9,10-dihydro-9,9-dimethylacridine	6-heptyltetrahydro-2H-pyran-2-one
3-methylbenzaldehyde	tetrahydro-6-pentyl-2H-pyran-2-one
undecylbenzoate	2-isobutylthiazole
n-butyl methyl sulfide	3-methyl-1-penten-4-yn-3-ol
cyclododecane	4-cyanocyclohexene
nonyl-cyclopropane	4-heptanone
diphenylamine	ethyl (all-Z)-5,8,11,14-eicosatetraenoate
2-(methylthio)-ethanol	5-hexenoic acid
9-octylheptadecane	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
hexane	n-acetyl-2,4-difluoroaminobenzene
hexatriacontane	benzaldehyde
7-hydroxy-4-methylchromen-2-one	p-tolualdehyde
L-methioninol	benzophenone
oleic acid	benzyl methyl ketone
pentanoic acid	4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2ol
phenol	(1S) 4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2-one
2-methyl-1-(methylthio)-propane	bicyclo(3.2.0)hepta-2,6-diene
propylpropanedioic acid	(1S-endo)(4,7,7-trimethyl-3 bicyclo [2.2.1] heptanyl) acetate
2-ethenyl-6-methylpyrazine	1-methylpropyl butanoate
tributyl phosphate	butylated hydroxyanisole
	(1R-4R-6R-10S)-9-methylene-4,12,12,-trimethyl-5-oxatricyclo(8.2.0.0)4,6 dodecane
	(E) 6-(2-butenyl)-1,5,5-trimethylcyclohexene
	diacetamide
	dodecanoic acid
	furan
	furfural
	hexanal
	hexanoic acid
	methoxyphenyloxime
	3a,6,6,9a-tetramethyl-1,4,5,5a,7,8,9,9b-octahydro-benzo [E] benzofuran-2-one
	n-decanoic acid
	octadecane
	pentadecanoic acid
	polyparaben
	propanoic acid
	2-methyl-3-hydroxy-2,4,4-trimethylpentyl propanoic acid
	tridecanoic acid
	Z-11-tridecen-1-ol acetate
	Z-8-methyl-9-tetradecenoic acid

*Note: Retention Times and CAS numbers for each compound are located in APPENDIX I.

Table 6. Organic compounds and retention times detected in the urine of both red wolf and gray wolf female canid species.

Retention Time [RT]	Compound
1.2679	acetic acid
1.7351	2-pentanone
1.9993	methyl propyl sulfide
7.0972	2-heptanone
9.4022	4-methyl-2-heptanone
14.5312	2-hydroxy benzaldehyde
15.7852	acetophenone
17.9056	nonanal
24.2749	quinolone
25.132	1,3-bis(1,1-dimethylethyl)-benzene
26.6629	nonanoic acid
27.5535	2-methyl-quinoline
33.4324	2-methyl-8-quinolinol
36.8287	2,4-bis(1,1-dimethylethyl)-phenol
37.274	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-
46.5078	tetradecanoic acid
52.6647	Z-11-hexadecenoic acid
53.5103	n-hexadecanoic acid
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide
58.9301	(Z,Z)-9,12-octadecadienoic acid
59.1043	(E)-9-octadecenoic acid
59.8669	octadecanoic acid

Figure 2 – Reconstructed ion chromatograms from GC/MS analysis of volatile compounds found in the urine of male gray wolf and female gray wolf

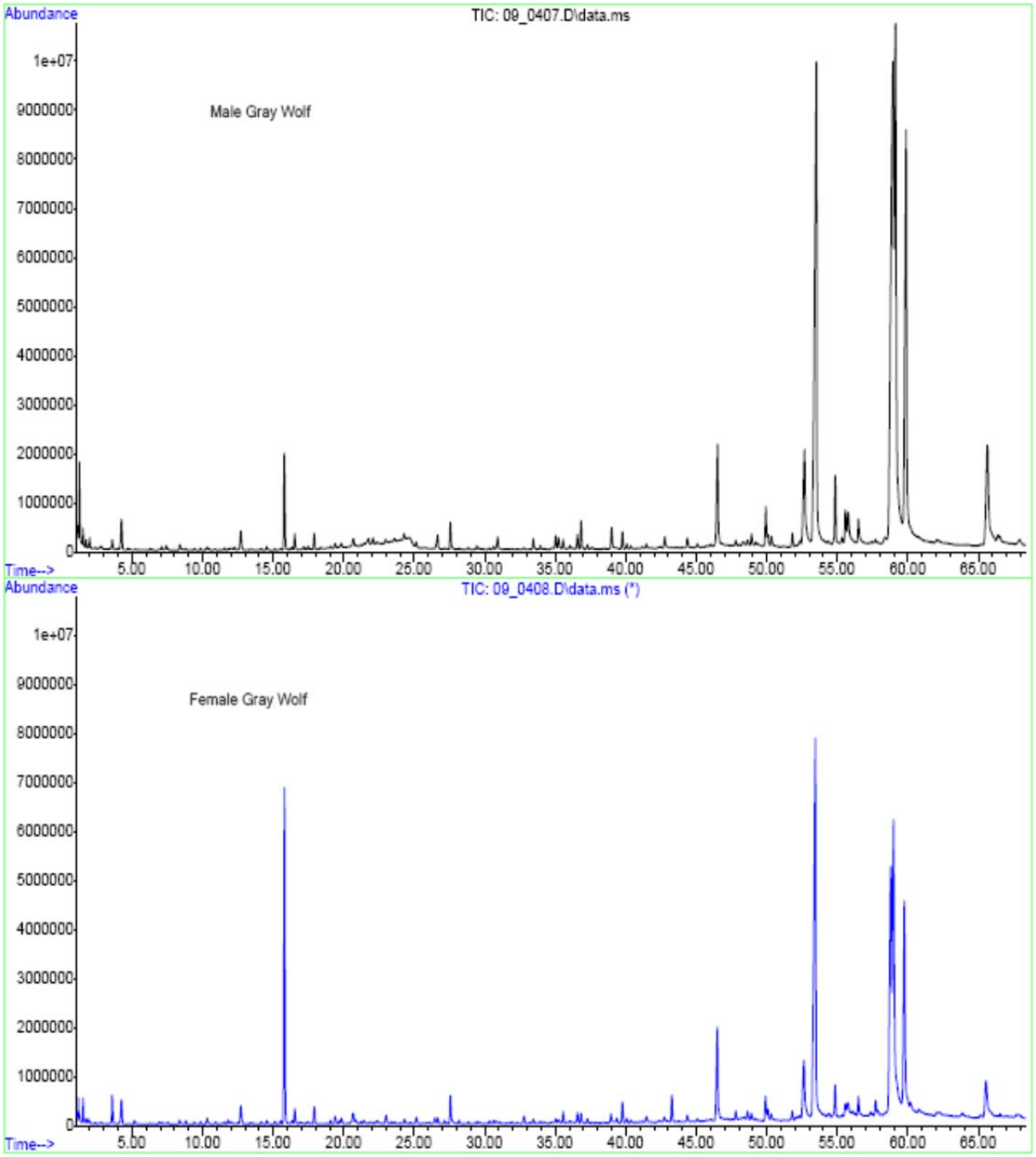

Table 7. Organic compounds found in both gray wolf male and gray wolf female urine

Retention Times [RT]	Compound
1.2679	acetic acid
1.5251	1-butanol
1.7351	2-pentanone
1.9185	propanoic acid
1.9993	methyl propyl sulfide
4.6884	furfural
5.2479	diacetamide
6.2481	4-heptanone
7.0972	2-heptanone
8.372	methoxyphenyloxime
9.4022	4-methyl-2-heptanone
10.317	benzaldehyde
10.8454	2-nitro-1,4-benzenedicarboxamide
14.5312	salicylaldehyde
15.1323	2-methyl-2-hexanol
15.7852	acetophenone
16.5213	p-tolualdehyde
17.9056	nonanal
25.132	1,3-bis(1,1-dimethylethyl)-benzene
26.6629	nonanoic acid
27.5535	2-methyl-quinoline
28.1672	(E,E) 2,4-decadienal
29.5943	1-methylpropyl butanoate
30.9198	n-decanoic acid
33.4324	2-methyl-8-quinolinol
36.0362	tetrahydro-6-pentyl-2H-pyran-2-one
36.8287	2,4-bis(1,1-dimethylethyl)-phenol
37.274	dihydroactinidiolide
38.9953	dodecanoic acid
41.1676	benzophenone
41.4502	polyparaben
42.7561	tridecanoic acid
44.362	6-heptyltetrahydro-2H-pyran-2-one
46.5078	tetradecanoic acid
49.9306	pentadecanoic acid
51.8237	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
52.6647	Z-11-hexadecenoic acid
53.5103	n-hexadecanoic acid
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide
58.9301	(Z,Z)-9,12-octadecadienoic acid
59.1043	(E)-9-octadecenoic acid
59.8669	octadecanoic acid
65.6431	ethyl (all-Z)-5,8,11,14-eicosatetraenoate

***Table 8.** Organic compounds detected in urine specific to male and female gray wolf

Male Grey Wolf	Female Grey Wolf
17-pentatriacontene	α pinene
1-octadecene	γ dodecalactone
dihydro-5-pentyl-2(3H)-furanone	1-(2,4-dimethyl-furan-3-yl)-ethanone
2-propylthiazole	1,2-benzenedicarbonitrile
3,3-dimethyl-5-phenyl-3H-pyrazole	1-nonene
3-amino-2-cyclohexenone	2,3-octanedione
3-octanone	2-isobutylthiazole
benzoic acid	3-methyl-1-penten-4-yn-3-ol
n-butyl methyl sulfide	4-cyanocyclohexene
butyl butyrate	5-hexenoic acid
1,2,3,4,5-pentamethylcyclopentene	n-acetyl-2,4-difluoroaminobenzene
1-cyclopropylpentane	benzyl methyl ketone
E,E-2,13-octadecadien-1-ol	4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2ol
heptadecanoic acid	(1S) 4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2-one
heptanoic acid	bicyclo(3.2.0)hepta-2,6-diene
imidodicarbonic diamide	(1S-endo)(4,7,7-trimethyl-3 bicyclo [2.2.1] heptanyl) acetate
n-formyl-imidodicarbonic diamide	butylated hydroxyanisole
methyl cyclohexane	(1R-4R-6R-10S)-9-methylene-4,12,12,-trimethyl-5-oxatricyclo(8.2.0.0)4,6 dodecane
pentanoic acid	(E) 6-(2-butenyl)-1,5,5-trimethylcyclohexene
2-propylmalonic acid	furan
n-(2-methylpropylidene) hydroxylamine	hexanal
undecanoic acid	hexanoic acid
Z-2-octadecen-1-ol	3a,6,6,9a-tetramethyl-1,4,5,5a,7,8,9,9b-octahydro-benzo [E] benzofuran-2-one
	octadecane
	2-methyl-3-hydroxy-2,4,4-trimethylpentyl propanoic acid
	quinoline
	Z-11-tridecen-1-ol acetate
	Z-8-methyl-9-tetradecenoic acid

*****Note: Retention times and CAS numbers for each compound can be found in APPENDIX I.

***Table 9.** Gender based relationships in organic compounds detected in male and female red wolf urine profiles

Male and Female Red Wolf Shared Compounds	Gender Specific Red Wolf Male Compounds	Gender Specific Red Wolf Female Compounds
acetic acid	propanoic acid	2,3-butanedione
2-pentanone	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine	2-methyl-1-(methylthio)-propane
methyl propyl sulfide	furfural	3-hexanone, 2-methyl
n-butyl methyl sulfide	3-methyl-2-hexanone	2,4-dimethyl-3-pentanone
2-heptanone	4-heptanone	2-(methylthio)-ethanol
4-methyl-2-heptanone	methoxyphenyloxime	L-methioninol
pentanoic acid	2,4-diphenyl-2,3-dihydro-1,5-benzothiazepine	3-ethylcyclopentanone
2-hydroxy benzaldehyde	benzaldehyde	phenol
acetophenone	1,4-benzenedicarboxamide, 2-nitro-	3-methoxy-2-butonal
nonanal	3-octanone	2-ethenyl-6-methylpyrazine
quinolone	cyclohexanone, 2,2,6-trimethyl-	hexane
1,3-bis(1,1-dimethylethyl)-benzene	benzaldehyde, 4-methyl-	1,2-diethiepane
2-methyl-quinoline	3,5-dimethyl-2-octanone	3-methylbenzaldehyde
3-methyl-2-heptanone	2-nonanone	propylpropanedioic acid
2-methyl-8-quinolinol	benzoic acid	nonyl-cyclopropane
2,4-bis(1,1-dimethylethyl)-phenol	hexanoic acid	(R) 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone
nonanoic acid	decanal	7-hydroxy-4-methylchromen-2-one
tetradecanoic acid	3,4-dimethyl-2-hexanone	diphenylamine
Z-11-hexadecenoic acid	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	tributyl phosphate
n-hexadecanoic acid	dihydro-3-pentyl-2(3H)-furanone	cyclododecane
(Z,Z)-9,12-octadecadienoic acid	1,1'-bicyclopentyl-1,1'-diol	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol
2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide	isobutyl isobutyrate	3,6-dihydro-3-hydroxy-6-(1-methylethoxy)-2H-pyran-2-methanol
(E)-9-octadecenoic acid	n-decanoic acid	9,10-dihydro-9,9-dimethylacridine
octadecanoic acid	ethanamine, 2-(dicyclohexylphosphino)-n,n-diethyl-	6,7-dimethoxy-2H-1-benzopyran-2-one
2,4-bis(1,1-dimethylethyl)-phenol	oxazolidine, 3-ethyl,2,2-dimethyl-	oleic acid
	benzeneamine, n-ethyl-2-methyl-5-nitro-	hexatriacontane
	tetrahydro-6-pentyl-2H-pyran-2-one	9-octylheptadecane
	n-(3,4-difluorophenyl)acetamide	undecylbenzoate
	3,3-dimethylpyrrolidine-2,5-dione	
	benzoic acid, 4-ethoxy-, ethyl ester	
	dodecanoic acid	
	benzophenone	
	tetrahydro-4,6-dimethyl-2H-pyran-2-one	
	2,4-dimethylundecane	
	6-heptyltetrahydro-2H-pyran-2-one	
	1-octadecanethiol	
	pentadecanoic acid	
	tetratriacontane	
	3-methylpentadecane	
	heptadecanoic acid	
	17-pentatriacontene	
	ethyl (all-Z)-5,8,11,14-eicosatetraenoate	
	3,5-dimethyl-2-octanone	

*****Note: Retention times and CAS numbers for each compound can be found in APPENDIX I.

Discussion

Many studies of the volatile chemical compounds in canid-urine have been conducted since the 1980s. These have highlighted the importance of identifying urinary chemical compounds for interpreting behaviors on both interspecies and intraspecies specific levels. Many intraspecific studies have focused on compounds found in both urine and anal gland secretions associated with reproduction (Asa, et al., 1990; Jorgenson, et al., 1978; Raymer, et al., 1984), genetic monitoring (Hausknecht, et al., 2007; Valiere & Taberlet, 2000), nutrition (Childs-Sanford, 2005; Darnell, et al., 2005; Delgiudice, et al., 1987), and scent-marking (Bowen & Cowan, 1980; Paquet, 1991; Peters & Mech, 1975; Rothman & Mech, 1979; Vila, et al., 1994; Zub, et al., 2003) while several interspecies related studies look at the dynamics of predators on prey related behaviors in response to canid urine (Christianson & Creel, 2010; Conover, 2007; Melchiors & Leslie, 1985; Taylor & Pekins, 1991).

Here we have identified various chemical compounds among canid species of both sexes that could provide information important to understanding both intraspecific and interspecific behaviors. Several compounds identified in both the male and female canid profiles have also been found in other carnivore urine chemical profiles, including acetic acid (Zhang, et al., 2005), 2-pentanone (Andersen & Vulpius, 1999), 2-heptanone (Andersen & Vulpius, 1999; Wood, et al., 2009; Zhang, et al., 2005), acetophenone (Jorgenson, et al., 1978; Zhang, et al., 2005), and nonanal (Andersen & Vulpius, 1999; Zhang, et al., 2005). Although the quantities of individual compounds were not assessed in this study, several studies have shown that even when compounds are found in both sexes, chemical quantities can be gender specific and may therefore convey sex-specific information. For example, levels of 2-heptanone in grey wolf males are elevated during the breeding season (Raymer, Wiesler et al., 1984). Similarly, acetophenone abundance is greater in the urinary profile of female ferrets compared to males (Zhang, et al., 2005) and female gray wolves compared to males (Raymer, Wiesler et al., 1984). As several compounds were found in both

sexes and in multiple carnivores, these chemical cues may change seasonally and may be associated with basic mammal physiology and reproduction.

One specific compound of interest identified in the canid profiles, heptadecanoic acid, was found to be unique to males across all four species. The same compound was also found to be one of the major constituents in male tiger urine-marking fluid (Burger, et al., 2008), suggesting that this chemical could be a significant male-specific compound across a broad taxonomic range (of mammals). Although the information capacity of a single compound is limited (Wilson & Stevenson, 2006), single compound signals are not unheard of, as several studies have shown that single compounds can elicit specific behavioral responses. For example, a single volatile chemical, trimethylthiazoline (TMT), found in fox urine has been shown to induce fear-responses in rats and mice, and the single chemical undecane has been identified as an alarm pheromone in carpenter ant (*Camponotus*), inducing aggressive ant behaviors (Wilson & Stevenson, 2006). In any case heptadecanoic acid is a conserved chemical that clearly carries information about carnivore sex and a behavioral study of this compound would be interesting.

In continuing with compounds associated with male canid profiles, several studies of urine in other male carnivores have also been conducted to include the tiger (Burger, et al., 2008), the fox (Jorgenson, et al., 1978); the ferret (Zhang, et al., 2005), the lion (Andersen & Vulpius, 1999), and the wolverine (Wood, et al., 2009). Comparison of compounds found in all four canid species and in those of the tiger, ferret, lion, and wolverine included 2-heptanone and benzaldehyde. Compounds found in all four canid species and in the tiger, ferret and lion included nonanal and those found in all four canids and the ferret included acetic acid and acetophenone. Compounds found in all four canid species profiles and those of the fox, ferret, the lion, and wolverine include 4-heptanone and those found in all four canids and just the fox and the ferret included quinoline, 2-methyl. The importance of these comparisons illustrates similarities in urinary profiles of male carnivores that may provide useful information in determining certain male associated behaviors or those marking behaviors that may impact predator/prey dynamics.

Furthermore, one study conducted on the male tiger (Burger, et al., 2008) specifically focused on compounds directly associated with scent-marking and a not a complete urinary chemical profile. As canids are well known to scent-mark to establish and maintain territories, we compared the tiger scent-marking chemical profile to those of the four canid species. We find the gray wolf exclusively shares one compound (nonan-4-olide), the red wolf shares one compound (hexanoic acid), the wolf-dog hybrid one (octanoic acid), and the domestic dog shares two compounds (decan-4-olide and (Z)-6-dodecan-4-olide) exclusively with the tiger. In addition, there are a range of compounds shared by the tiger and one or more of the male canids to include n-decanoic acid, heptanoic acid, and undecanoic acid. The pattern is interesting– (why the variation in chemicals shared between male canids and tigers?) but not particularly informative as there is no obvious pattern with respect to specific scent-marking compounds.

When comparing all canid urine compounds, not just male-specific compounds, with those of the tiger (Burger, et al., 2008), we find multiple similarities to include nonanoic acid; tetrahydro-6-pentyl-2H-pyran-2-one; dodecanoic acid; 6-heptyitetrahydro-2H-pyran-2-one; tetradecanoic acid; pentadecanoic acid; and n-hexadecanoic acid. As urinary marks from female tigers were not analyzed in the comparison study and many of these compounds are also found in the female canid urinary profiles in this study, it is unclear whether these compounds are chemical cues associated with sex or whether they are providing other information.

This study provided the first urine chemical profile for the red wolf, and these were compared to the gray wolf. We found that grey wolf urine contained 94 organic compounds with only forty-three compounds shared between the sexes, another twenty-three found only in males and twenty-eight in only females. For red wolves, 96 identified organic compounds were identified, with forty-two compounds being male specific, thirty compounds female specific and only twenty-three compounds were shared by the sexes. Thus male and female grey wolves share more urinary chemical compounds than red wolf males and females.

Both species of wolf share nineteen compounds across the sexes, while males share an additional seven chemicals between one another, and females share an

additional two chemicals between one another. Grey wolf females also share one compound, pentadecanoic acid, specifically with the red wolf male and both the red wolf male and female share two compounds n-butyl methyl sulfide and pentanoic acid specifically with the grey wolf male. The number of chemical differences between the red wolf male and female profiles is greater than those between grey wolf male and female profiles which warrant's further investigation. It is unknown as to whether this compound difference significantly impacts the behavior of the red wolf versus that of the grey wolf. In addition, further analysis of red wolf urine chemical profiles and their gender differences through behavioral studies may prove useful in countering management concerns of hybridization of red wolf females with coyote (*Canis latrans*) males.

As this study provides the first urine chemical profile for the wolf-dog hybrid, we compared it to the profiles for both the male and female gray wolf and the male domestic dog (female dog profiles were not measured in this study). We found several differences between the parental taxa and the hybrid. The wolf-dog hybrid profile revealed seventeen compounds found in neither the male or female grey wolf nor the male domestic dog. As domestic dog female samples were unavailable for this portion of the study, it is important to note that some of these compounds may be found in the urinary profile of the domestic dog female. However if they are not present in the urinary profile of the domestic female dog, this indicates that there could be gene-gene interactions that have generated new chemicals not found in the urine of the parental taxa. Hybrids often have phenotypes that are not intermediate to the parentals, and at times extreme phenotypes can be produced that are beyond that of parental taxa (Wolf, 2000). In any case, there are clearly epistatic effects influencing urine chemicals in the wolf-dog hybrid, and it should be noted that the potential for interactions like this is considerable (Wolf, 2000).

Using the SBSE method coupled with GC/MS, 144 organic compounds were identified for male canids here, with thirty-one of the identified compounds being found in the males of all four species. The same technique identified 102 organic compounds in female canids with twenty-two of the identified compounds being the

same in the females of both species. As expected, if we compare our findings to previously published gray wolf and domestic dog chemical urine profiles there are many similarities. The only profile consistencies between the previous studies for both the gray wolf and domestic dog included 2-pentanone, furfural, 4-heptanone, 2-heptanone, benzaldehyde, acetophenone, and nonanal (Raymer, 1984). Those associated only with the domestic dog include methyl isobutal ketone; 3-ethylcyclopentanone; benzonitrile and those associated only with the gray wolf included methyl butyl sulfide and methyl propyl sulfide (Raymer, 1984). We also saw several differences in which the compounds identified in this study were expressed in different species from those in previous studies of wolf and dog urine profiles. For example, in this study we found 3-octanone in the gray wolf but not in the domestic dog whereas, previous studies found 3-octanone in the domestic dog but not in the gray wolf (Raymer, 1984).

It is important to point out that different analytical methods and sample preparation procedures can influence compound recovery from biological samples (Mitra, 2003; Soini, et al., 2005; Zhang, et al., 2005), which may account for some of the differences in compositions of the chemical profiles in this study compared to those in previous studies. Biological secretions include a myriad of chemical compounds to include alcohols, aldehydes, carbohydrates, esters, fatty acids, ketones, and phenols where detection depends on individual chemical properties making it difficult to find a method that is optimal for detecting them all (Andersen & Vulpius, 1999).

In addition, samples in this study were pooled similar to the 1984 study by Raymer. However, it is important to note that running all samples independently would provide additional variations between groups to include individuals, sexes, and species, therefore potentially providing additional chemical compounds. Independent analysis would also enhance the comparison of compounds across and within canid groups by allowing for additional statistical analysis further strengthening similarities and differences between groups.

In spite of the numerous behavioral studies implicating urine as a semiochemical source, there is very little known about the impact individual chemicals have on

behaviors (Albone, 1984). Because semiochemical signals are often conveyed in complex mixtures, the number of possible messages received and how animals ascertain the meaning of each signal is large, which makes it difficult to determine which chemical or combination of chemicals is responsible for eliciting a particular behavior. In addition, complex signals may also be less reliable than single compound signals as background odors may interfere with the information content or concentration levels of chemicals may vary, which can impact the information conveyed (Wilson & Stevenson, 2006). However, more complex mixtures are able to carry more information than simple messages, this may be more difficult to misinterpret and hence could be favored by selection for these reasons. In any case, there is a need for more chemically based behavioral studies that focus on identifying responses to urine chemical profiles and to individual urine chemicals.

It is the differences and similarities of the chemical profiles among this study and previous studies that may prove useful to canid biologists in identifying specific chemosensory signals important to reproduction, territory maintenance, scent-marking or even the development of behavior modification tools to assist in predator and/or prey management schemes. Additionally, the analysis of both the red wolf and wolf-dog hybrid urines provide chemical profiles for two canids that have not been analysed in previous studies, and this work shows that epistatic interactions seem partly responsible for hybrid profiles.

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Chapter Four

Impact of Select Volatile Organics from Canid Urinary Profiles on Scent-Marking Behaviors of Wolves

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ABSTRACT

Many animals use olfactory signals to convey information to conspecifics and other animals. These chemical messages are often found in and released through biological fluids such as urine. Using select chemicals and chemical combinations from the urinary profiles of canids, two species of wolf - the gray wolf (*Canis lupus*) and the red wolf (*Canis rufus*) - were observed to determine the impact of these chemicals on scent-marking behaviors. One chemical combination, CAM (control/acetophenone/methyl-propyl sulfide), was found to increase the number of raised-leg urinations in the males of both species indicating that certain chemicals in canid urine impact scent-marking behaviors.

Introduction

The identification of exclusive cues which trigger specific behavioral responses in animals has frequently proved challenging to ethologists, as has the interpretation of the behaviors elicited (Blomquist & Bagneres, 2010)), but ascertaining cues and the meaning of the responses they elicit has become increasingly important for wildlife management. Two theories once dominated the study of animal behavior stating that behaviors must be either learned or innate (Abrantes, 2005). Over time, it has become clear that animal behavior is more complex than this simply dichotomy suggests, and consists of a combination of learned behaviors, innate behaviors, genes which can encode certain behaviors, and an animal's interaction with the environment (Mori et al., 1995; Abrantes, 2005; Touhara and Vosshall, 2009; Hayden et al., 2010; Ingleby et al., 2010). Some of this change resulted from the work by Konrad Lorez, Nikolaas Tinbergen, and Karl Von Frisch, which resulted in the 1973 Nobel Prize, which identified four ways in which genes encode for behaviors, including "sign stimuli", visual or olfactory signals that triggers a specific stereotyped behavior (Abrantes, 2005).

Since the discovery of "sign stimuli", multiple studies have been conducted on a variety of animals from insects (Moore, 1997; Sharma, et al., 2012; Ingleby, et al., 2013) to mammals (Anisko, 1976; Melchiors & Leslie, 1985; Zhang, et al., 2005; Parsons, 2010) in order to identify and isolate visual and olfactory cues (chemosensory signals) that trigger specific stereotyped behaviors. These studies provide valuable information generally, and have been used to inform wildlife management. For example, studies have identified that the chemosensory signals in predator scats can be employed as repellents for problematic prey species like black-tailed deer (Melchiors and Leslie, 1985) and kangaroos (Parsons and Blumstein, 2010), and more recently a study of chemosensory signals in urine-marks is identifying chemicals to create an experimental bio-fence to facilitate the behavioral management of African wild dogs (Apps et al., 2012).

Another interesting example of "sign stimuli" and the relationship between chemosensory signals and stereotypical behavior can be seen in territory

maintenance by large predators, including wolves. Wolves, like many canids, scent-mark territory boundaries to establish and signify occupied space. There are a range of stereotypic behaviors associated with scent-marking, including sniffing (Peters and Mech, 1975; Albone, 1984; Wyatt, 2003), defecation (Peters and Mech, 1975; Nunez and Miguel, 2004; Childs-Sanford, 2005; Rabon, 2009), scratching/digging (Peters and Mech, 1975; Nunez and Miguel, 2004; Rabon, 2009), rubbing, and urine-marking (Peters and Mech, 1975; Asa, et al., 1990; Wyatt, 2003; Nunez and Miguel, 2004; Rabon, 2009). The detection of these behaviors can be used to signify animal responses to sign stimuli.

Urine contains a plethora of chemical signals conveying a host of different messages that impact animal behavior, and is commonly used in scent-marking behavior. The information contained in urine depends on whether the animal is simply excreting biological waste or scent-marking (leaving chemical signals for other animals). One of the easiest ways to discriminate between these two classes of urination and to identify when olfactory chemical messages are being conveyed is to use the body postures of the urinating animal. For example, when urinating, wolves adopt a variety of postures to include raised-leg urination (RLU), squat urinations (SQU), and standing-urinations (STU). Previous studies have shown that RLUs are the primary method of urine marking in males and urine excreted this way will incite a high rate of sniffing by both males and females, whereas SQUs and STUs on the other hand are associated more with simple waste excretion and less frequently elicit behavioral responses from conspecifics (Peters and Mech, 1975; Nunez and Miguel, 2004). Thus body posture can be used as a proxy for the “intent” of the urinating animal. Other behaviors tied to urine-marking events include the animal’s behavior in the presence of a pre-existing chemosensory signal. Existing signals often trigger scent-marking events and can be identified by several distinct and repetitive behavior patterns that include sniffing of an area and then urinating directly over it, ground scratching after urination, and urinating or over-marking an area where a previous urine deposit was left (Bekoff and Wells, 1986; Abrantes, 1997). Understanding the “sign stimuli” and the

stereotypical behaviors associated with scent-marking can assist in species management and the development of behavioral modification tools.

Although multiple studies have been conducted using urine and chemosensory signals to determine their impact on wolf behavior, from the number of times a pack scent-marks its territory (Peters & Mech, 1975), to various reproductive strategies (Raymer, et al., 1984; Asa, et al., 1990; Packard, 2003; Rabon, 2009), few have looked at the individual chemicals in urinary profiles of wolves to determine those specifically associated with scent-marking behaviors. Here, the impact of several chemicals (acetophenone and methyl propyl sulfide [MSP]) associated with scent-marking behaviors in tigers (Burger, et al., 2008), foxes (Jorgenson, et al., 1978), ferrets (Zhang J. , et al., 2005), lions (Andersen & Vulpius, 1999), and wolverines (Wood, et al., 2009), were investigated to determine their impact on wolf scent-marking behaviors including urine-marking, sniffing, defecation, scratching/digging, and rubbing. These compounds are found in both gray wolf (*Canis lupus*) and red wolf (*Canis rufus*) urinary profiles at retention times 15.7852, 1.9993, and 10.317 respectively (Appendix I) and are believed to influence the scent-marking behavior of wolves.

Materials and Methods

Study Sites and Subjects

Permissions were obtained from both the SSP (Species Survival Plan) Coordinator for the endangered red wolf and the Fort Worth Zoo in Fort Worth Texas to use their animals in the behavioral study. The site contained a pair of male and female animals. The red wolf study site was located at the Fort Worth Zoo in Fort Worth, Texas. The gray wolf study site was located in a remote area of Wales, England, at Wolf Watch UK a private captive wolf facility and contained two enclosures (one contained a male and female and the second contained two females).

Scent Stations

The scent stations were located in areas animals frequently passed through to simulate territory boundaries. These stations were set up on the outside of each of

the captive animal enclosures to alleviate the natural curiosity and disturbance by the wolves. Each station consisted of four scent stakes (36" Garden Zone, U-Post Steel Light Duty, Model #090033) placed in a linear row, 1-ft away from the enclosure fence, 3-ft apart, and buried 5" into the ground. Stakes were selected to simulate higher substrates naturally used by wolves for territory scent marking and to facilitate chemical dispersion through the air column. Each individual stake contained one – ½" non-metallic one hole snap strap (Lamson & Sessions, Conduit strap, Model #E978DC-CAR) bolted to the second hole in each stake by a ¼"-20 x ¾" VP-round combo machine screw (Hillman Group, Model #35215). The conduit strap was then used to secure the various scent vials in the experiment.

Scent vials were designed using a single 12ml (27 x 36mm) Nalgene sample vial with a snap cap (Daigger, Model #EF28320C) with a single hole drilled through the cap to enable air flow. Each vial contained one sterilized cotton ball which was inoculated with 1ml of the appropriate chemical treatment prior to placement within the scent-stations. As compound quantities in whole urine were not available from the urinary profiles used to identify chemicals for the behavioral tests, a 1ml standard measurement was employed. The standard was selected to compensate for external variables, like weather, as chemicals were contained in an enclosed dispersal device limiting evaporation and test subject exposure. The primary chemicals used in the study included acetophenone (Sigma-Aldrich, catalogue #A10701-5ml, lot# MKBH7902V, 99% purity), methyl propyl sulfide (VWR, catalogue #AAAL09346-09, lot#10139856, 99% purity), and benzaldehyde (Sigma-Aldrich Company). These chemicals were investigated because they were found in the urinary profiles of all four canid species and in both male and female wolves and are associated with scent-marking events in other mammals including tigers, foxes, ferrets, lions, and wolverines. Water was used as a control at all three study sites. Whole urine samples were not used as a positive control due to restrictions at captive facilities concerning the possible transmission of zoonotic diseases. However, future studies of wild populations would benefit from a study using whole urine as a positive control. Chemicals were placed and rotated among the stakes every 7 days, throughout the duration of the study. The rotation allowed for

behavioral observation of wolves in response to individual chemicals as well as associations between these chemical. It is important to note that future studies should combine individual chemicals together in a single vial instead of placing multiple chemical vials containing a single chemical and note the behavioral impact of the single vial chemical combination. All chemicals placed at a station longer than 7 days were re-charged with 1ml of chemical.

All scent stations were monitored for several weeks prior to chemical placement to ensure that animal response and camera activations were due to the actual chemicals and not the novelty of the scent stations and human activity collecting digital feeds.

Camera Traps and Live Behavioral Observations

Due to budget limitations, a total of four camera traps (Moultrie 160 Digital Game Cameras) were used in the behavioral study. Two cameras were set up for red wolf observations at the Fort Worth Zoo study site outside the pack enclosure at the scent station. Camera one was set up from March 15, 2008 through February 28, 2009 for a total of 350 days continuously. Camera two was set up from August 9, 2008 through February 28, 2009 for a total of 203 days continuously. The observational design for red wolves included 150 days of observations in the absence of chemicals and 150 days of observations in the presence of chemicals. Due to equipment malfunction and other environmental conditions, only 105 days of observation occurred in the absence of chemicals and 127 days of observation occurred in the presence of chemicals. At the United Kingdom gray wolf study sites in Wales, one camera was placed at each pack enclosure. Due to the limited availability of the UK sites, both camera one and camera two were set up from June 3, 2008 through June 27, 2008 for a total of 24 days each. Observational design for gray wolves included 12 days of observations in the absence of chemicals and 12 days in the presence of chemicals. Due to equipment replacement and other environmental conditions, only 8 days of actual observations occurred in the absence of chemicals and 11 days of observation occurred in the presence of chemicals. All cameras were motion activated to

capture pictures as well as video feeds. Feeds were collected and dropped to a digital hard drive at least once per week at all study sites. Pictures and video feeds were later analyzed and behavioral observations recorded (Appendix II).

Live observations consisted of visual observations after chemical placement and included observations from site administrators as well as video recordings from hand held video cameras at various times throughout the study. All live observations were recorded in journal logs which were later analyzed and behavioral observations noted.

Behavioral Observation Analysis

Digital feeds, pictures, and journal logs were all used to identify specific behaviors regardless of whether the feed was pre- or post- chemical placement (Appendix III). The select behavioral characteristics pulled for analysis were those associated with scent-marking and included all urination events, defecation, scratching, digging, rubbing, sniffing, and licking within a 12-foot radius of the scent station. The behaviors were then cataloged and compared for differences in activity levels, scent-marking behaviors, and urine-marking events in the presence and absence of chemicals. The chemical treatment was used as a categorical grouping variable with activity levels, scent-marking behaviors, and urine marking events being dependent variables. For each treatment, chemical and control, activity levels and scent-marking behavioral responses by red wolves and by gray wolves were compared using a one-tailed z-test of proportions for direct counts. The behavioral changes between two treatments were compared using Chi-square. Scent-marking and urine-marking behavioral responses of red wolves and gray wolves to the CAM treatment and control were compared using a t-test, two-sample assuming unequal variances. Statistical analyses were conducted using Microsoft Office Excel 2010 software, according to Fowler et al. (1998) and statistical significance was set at $p \leq 0.05$.

Results

Results are presented by species for activity level, scent-marking, and urine-marking behaviors in the presence and absence of chemicals. Comparisons were not made across red wolf and gray wolf datasets due to the difference in experimental study length as a result of site availability (red wolf = 350 days; gray wolf = 24 days).

Behavioral Analysis - Red Wolves

Red wolves activated the camera trap 2688 times both in the presence and absence of chemicals. The wolves stopped at the scent-station perimeter 667 times and passed through the scent-station area without stopping a total of 2021 times. The number of camera activations by the wolves when the chemicals were absent was 776 with 397 activations passing through the scent-station area and 379 stopping in the scent-station area. The number of camera activations by wolves in the presence of chemicals was 1912 with 1624 activations passing through the scent-station area and 288 stopping in the scent-station area. In the presence of chemicals, the wolves exhibited a statistically significant increase in the number of times they passed through the scent-station area (z = 27.29; p < 0.001) (Figure 1) but a non-statistically significant decrease in the number of times they actually stopped (z = -3.52; p = 0.10) (Figure 1). The overall activity level recorded in the presence of chemicals showed to be statistically significant (X^2 (1, N = 2688) = 337.55; p < 0.001) suggesting that wolves were more active in the scent-station area during the chemical presence (Figure 1).

A total of 680 video camera and live observation feeds were collected and identified for behavioral analysis. Scent-marking behaviors were observed in 161 of these feeds and included 8 urine-marking events, 1 defecation event, 12 scratching/digging event, 14 rubbing events, and 97 sniffing events. Combinations of scent-marking behavior included 5 urine-marking/scratch/dig events (4-RLU; 1-STD), 14 urine-marking/sniffing events (11-RLU; 2-SQU; 1-STD), 2 urine-marking/scratching/digging/sniffing events (1-RLU; 1-STD) 5 sniffing/scratching/digging events, and 3 rubbing/sniffing events.

A total of 84 scent-marking observations occurred in the absence of chemicals and 77 occurred in the presence of chemicals producing a statistically significant difference (X^2 (1, N = 161) = 8.57, p = 0.003) (Figure 2). Although more scent-marking behaviors were observed in the absence of chemicals, a shift in the types of behaviors was noted between those recorded in the absence of chemicals versus those observed in the presence of chemicals. In the absence of chemicals, the wolves increased scratching, rubbing and sniffing (N = *Total Count* = 76) in the scent station area. With the addition of chemicals, wolves decreased scratching and rubbing behaviors (N = 56), but increased the number of urine-marking events (N = 8 chemical absence; N = 21 chemical presence) producing a statistically significant difference (z = 2.41, p = 0.008) (Figure 2) implying that some chemical message is being received by the wolves triggering a urine-marking response.

The chemicals inciting scent-marking behaviors in red wolves included combinations of the control (water), acetophenone, and methyl propyl sulfide. Both the CA (control/acetophenone) combination and the CAM (control/acetophenone/methyl propyl sulfide) produced a difference in scent-marking behaviors recorded in relation to the number of days the wolves were exposed to the chemical/chemical combinations. In the absence of chemicals (control), the red wolves exhibited 84 scent-marking behaviors over 105 days or a rate of 0.80 scent-marking events per day. The CA treatment incited 4 scent-marking behaviors over 5 days or a rate of 0.80 times per day and the CAM combination incited 43 scent-marking behaviors over 37 days or a rate of 1.16 times per day. The average scent-marking behaviors per day by wolves were higher in the presence of the CAM chemical (Mean = M = 1.65, Standard Deviation = SD = 1.09) then in the absence of chemicals (M = 2.4, SD = 2.20), the difference did not reach statistical significance (t (52) = 1.74, p = 0.09).

In further analyzing urine-marking behaviors, the study shows an increase in urinary events in the presence of the CAM chemical combination. In the absence of chemicals (control) only 8 urine-marking events (4-RLU; 3-SQU; 1-STD) were

recorded, while the presence of the CAM chemical treatment demonstrated an increase in urine marking events to 15 observations (14-RLU; 1-SQU). Although urine marking behaviors of the wolves increased in the presence of the CAM chemical treatment (*M* = 1.50, *SD* = 0.97) over those observed in the absence of chemicals (*M* = 1.33, *SD* = 0.52) it did not reach statistical significance (*t* (14) = -0.45, *p* = 0.66).

FIGURE 1. Activity levels of red wolves in the presence and absence of chemicals.

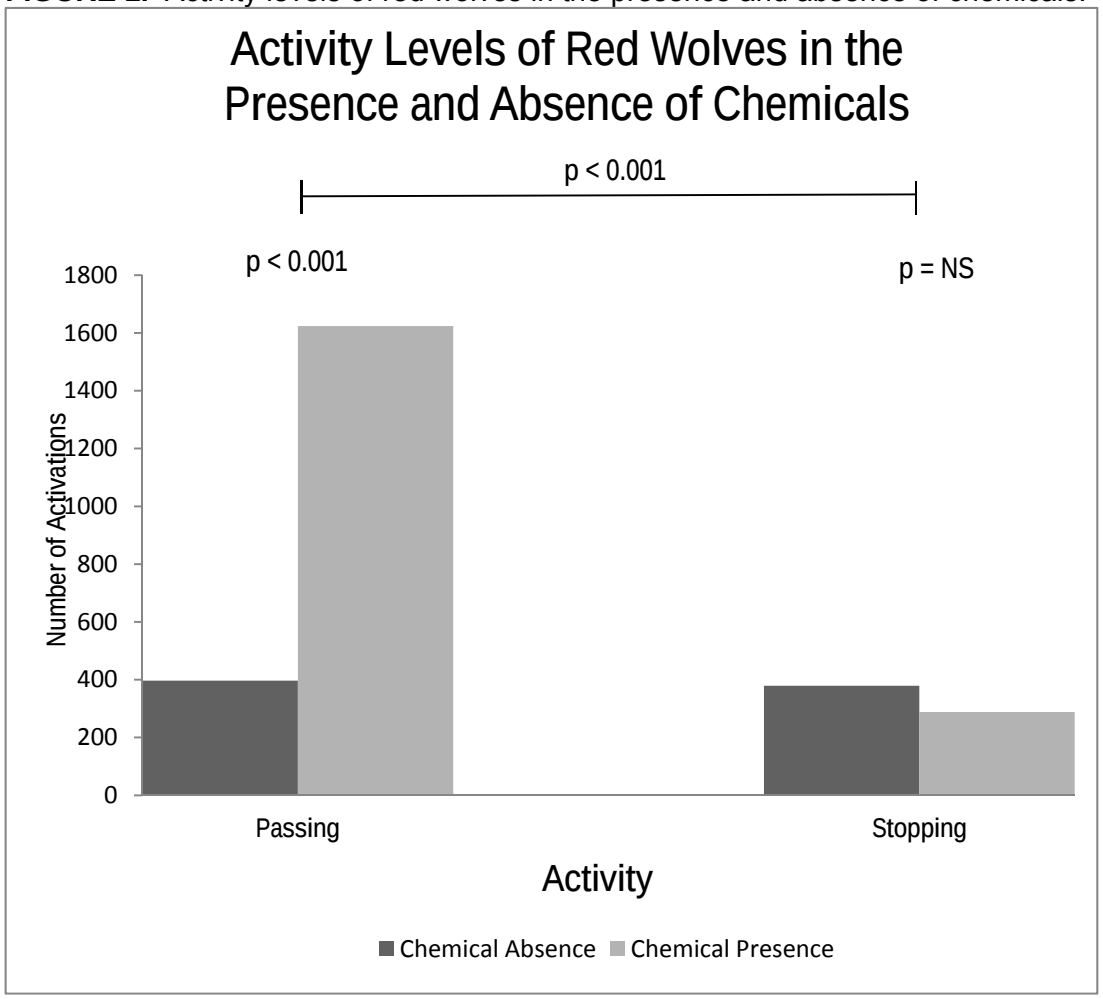

Figure 1. Number of camera activations by red wolves in the absence of chemicals as they passed through (N = 397) or stopped (N = 379) in the scent station area were compared to the number of camera activations in the presence of chemicals as red wolves passed through (N = 1624) or stopped (N = 288) in the scent-station area. The p-values below the lines are associated with comparisons of passing (left value) and stopping (right value) in the absence (dark bars) and presence (light bars) of chemicals, while the p-value above the line is associated with comparisons of overall changes in activity levels in the presence or absence of chemicals (NB there was more total activity with chemicals).

FIGURE 2. Scent-marking behaviors of red wolves in the presence and absence of chemicals.

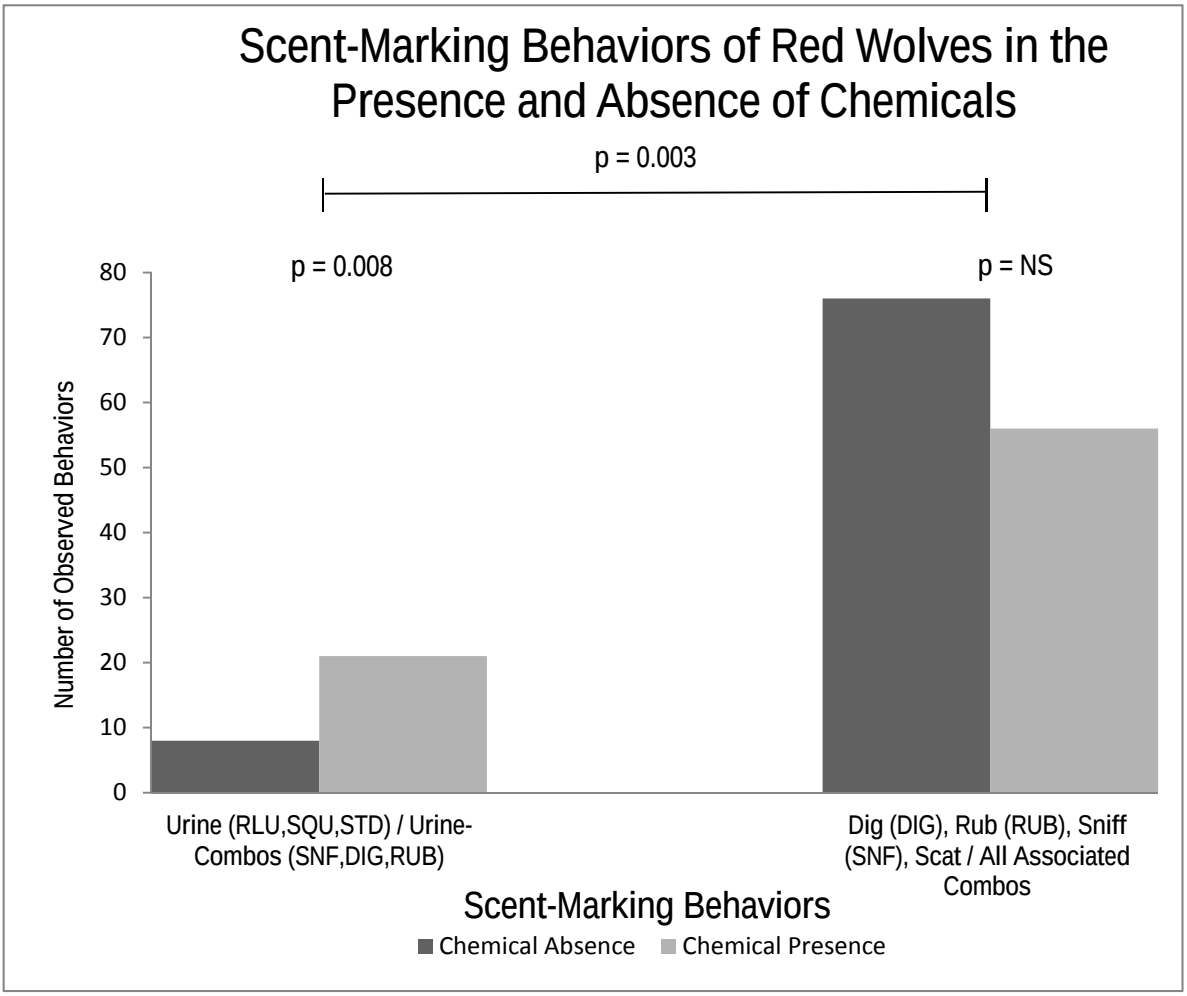

Figure 2. Scent-marking behaviors of red wolves fluctuated in the presence and absence of chemicals. Scratching, rubbing, digging, scat, and all associated combination events increased in the absence of chemicals while urine-marking events increased in the presence of chemicals. The number of scratching, rubbing, digging, scat, and all associated combination events by red wolves in the absence of chemicals (N = 76) were compared to the same events in the presence of chemicals (N = 56) as well the number of urine marking in the absence of chemicals (N = 8) to those in the presence of chemicals (N = 21). The p-values below the lines are associated with comparisons urine-marking events (left value) and scratching, rubbing, digging, scat, and all associated combinations (right value) in the absence (dark bars) and presence (light bars) of chemicals, while the p-value above the line is associated with comparisons of overall changes in scent-marking behaviors in the presence or absence of chemicals.

Behavioral Analysis - Gray Wolves

Gray wolves activated the camera trap 434 times both in the presence and absence of chemicals. The wolves stopped at the scent-station perimeter 90 times and passed through the scent-station area without stopping a total of 344 times. The number of camera activations by the wolves when the chemicals were absent was 185 with 152 activations passing through the scent-station area and 33 stopping in the scent-station area. The number of camera activations by wolves in the presence of chemicals was 249 with 192 activations passing through the scent-station area and 57 stopping in the scent-station area. In the presence of chemicals, the wolves exhibited an increase in the number of times they passed through (z = 2.16; p = 0.02) and stopped (z = 2.53; p = 0.006) in the scent-station area, both of which showed to be statistically significant (Figure 3). However, the overall activity level recorded in the presence of chemicals did not reach statistical significance (X^2 (1, N = 434) = 1.65, p = 0.20).

A total of 135 video camera and live observation feeds were collected and identified for behavioral analysis. Scent-marking behaviors were observed in 113 of these feeds and included 8 urine-marking events (5-RLU; 3-SQU), 1 rubbing event, 1 scratching/digging, and 91 sniffing events. Combinations of scent-marking behavior included, 9 urine-marking/sniffing events (8-RLU; 1-SQU), 1 sniffing/scratch/dig event, and 2 rubbing/sniffing events.

A total of 37 scent-marking behaviors occurred in the absence of chemicals, with 85 occurring in the presence of chemicals. Although a greater number of scent-marking behaviors were observed in the presence of chemicals it did not reach statistical significance (X^2 (1, N = 122) = 0.01, p = 0.93) (Figure 4). In the presence of chemicals, the wolves increased scratching, rubbing, and sniffing behaviors (N = 73) over those observed in the absence of chemicals (N =32) producing a statistically significant difference (z = 4.00; p = 0.001) (Figure 4) suggesting that chemical presence had some impact on scratching, rubbing, and sniffing scent-marking behaviors. The number of urine-marking events recorded for gray wolves also produced an increase in the presence of chemicals (N = 12) over those

observed in the absence of chemicals (N = 5), producing a statistically significant difference (z = 1.70; p = 0.04) suggesting that chemical presence also impacts urine-marking events.

The chemicals inciting scent-marking behaviors in gray wolves included combinations of control (water), acetophenone, and methyl propyl sulfide. In the absence of chemicals (control), the gray wolves exhibited 37 scent-marking behaviors over 8 days or a rate of 4.63 scent-marking events per day. Exposure to the CAM treatment incited 76 scent-marking behaviors over 11 days or a rate of 6.91 times per day. The average scent-marking behaviors per day by gray wolves were higher in the presence of the CAM chemical (M = 6.91, SD = 5.97) then in the absence of chemicals (M = 4.11, SD = 2.67), the difference did not reach statistical significance (t (14) = -1.39, p = 0.19).

In further analyzing specific scent-marking behaviors, the study shows an increase in urinary events in the presence of chemicals. In the absence of chemicals (control) only 5 urine-marking events (3-RLU; 2-SQU) were observed, while the CAM treatment showed a total of 12 urine marking events (9-RLU; 3-SQU). Although urine-marking behaviors of the wolves increased in the presence of the chemical treatment (M = 1.5, SD = 0.76) over those observed in the absence of chemicals (M = 1.25, SD = 0.50) it did not reach statistical significance (t (9) = -0.68, p = 0.51).

FIGURE 3. Activity levels of gray wolves in the presence and absence of chemicals.

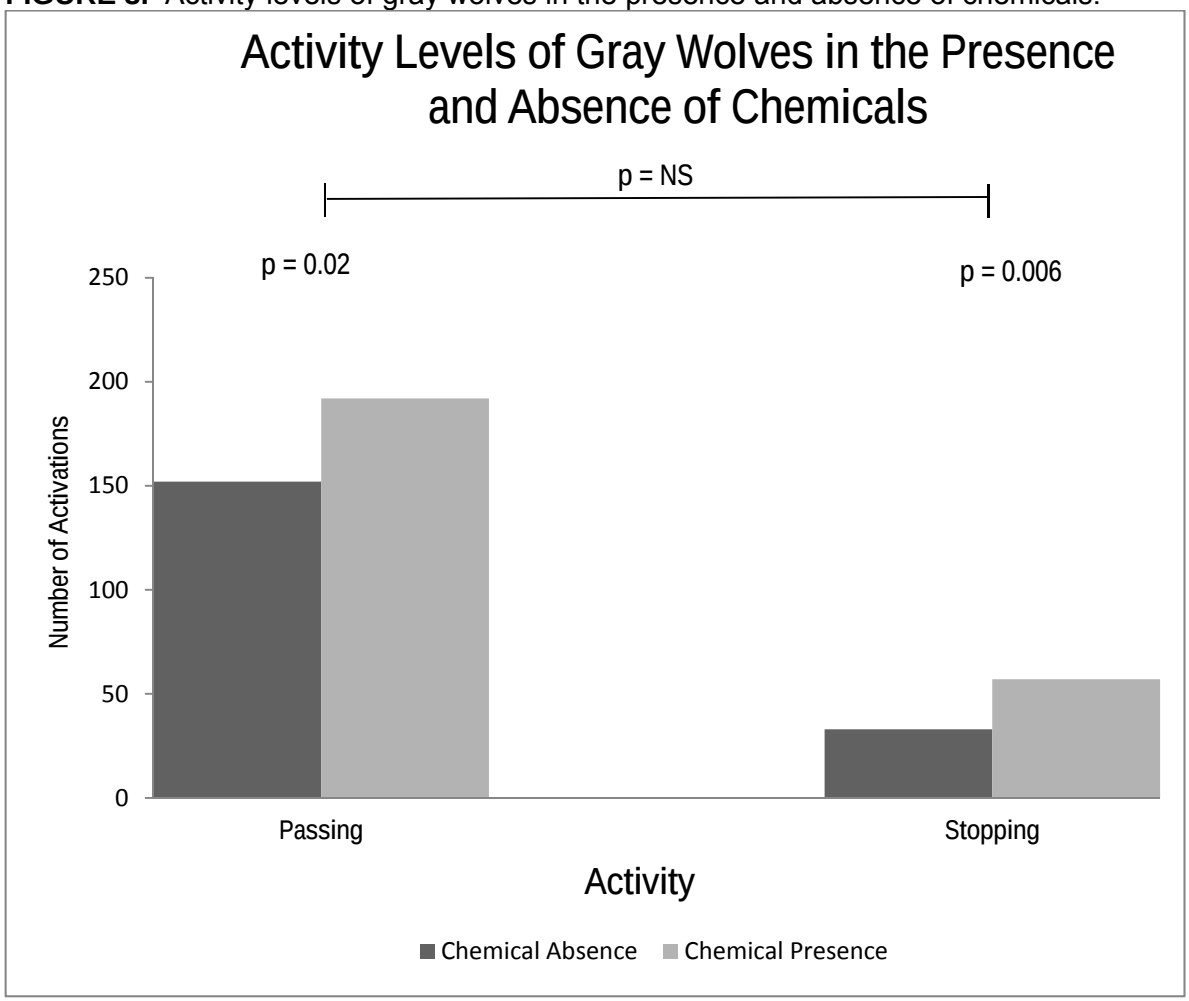

Figure 3. Number of camera activations by gray wolves in the absence of chemicals as they passed through (N = 152) or stopped (N = 33) in the scent station area were compared to the number of camera activations in the presence of chemicals as gray wolves passed through (N = 192) or stopped (N = 57) in the scent-station area. The p-values below the lines are associated with comparisons of passing (left value) and stopping (right value) in the absence (dark bars) and presence (light bars) of chemicals, while the p-value above the line is associated with comparisons of overall changes in activity levels in the presence or absence of chemicals.

FIGURE 4. Scent-marking behaviors of gray wolves in the presence and absence of chemicals.

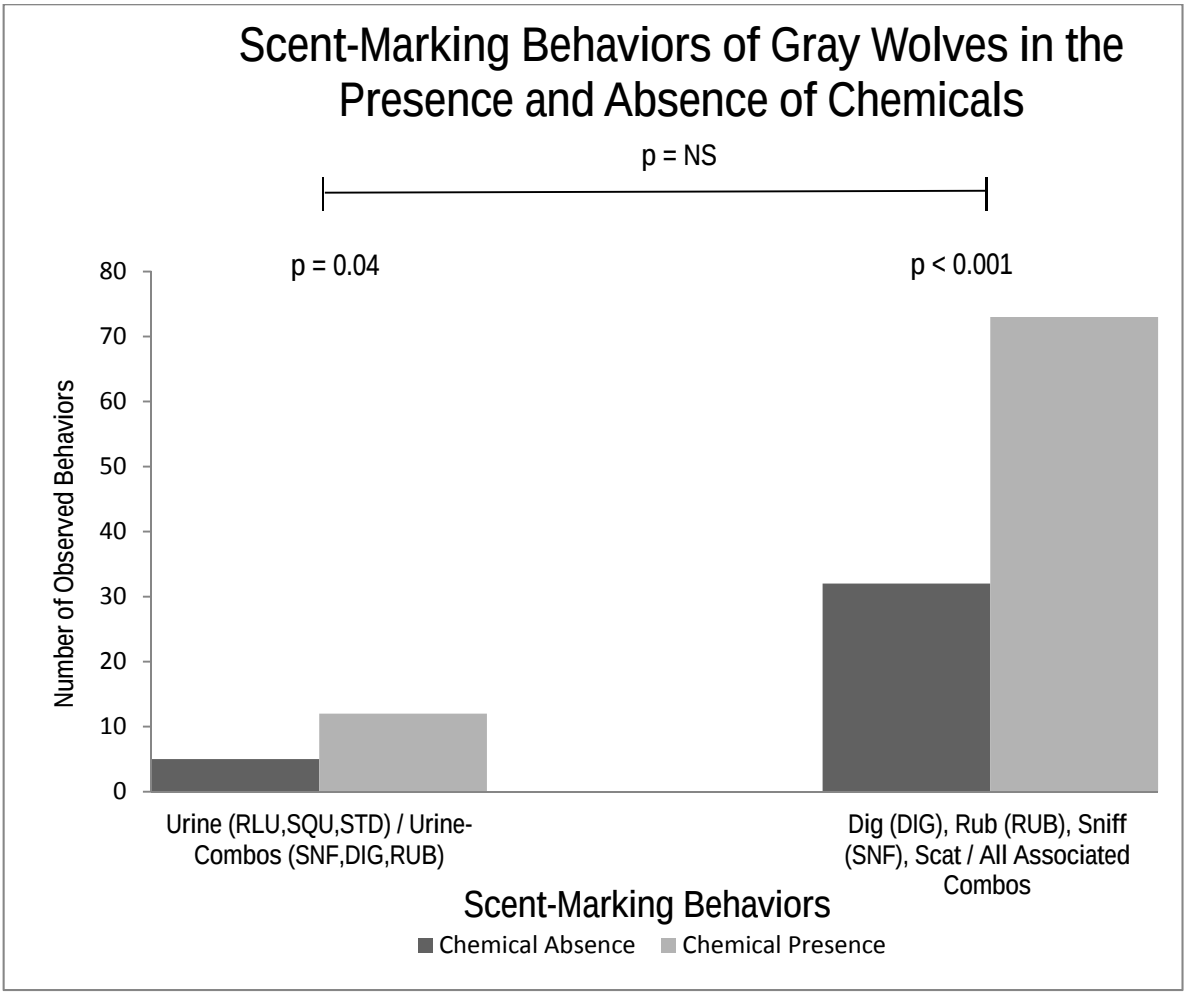

Figure 4. Scent-marking behaviors of gray wolves increased in the presence of chemicals in both urine-marking events as well as other scent-marking behaviors. The number of scratching, rubbing, digging, scat, and all associated combination events by gray wolves in the absence of chemicals (N = 32) were compared to the same events in the presence of chemicals (N = 73) as well as the number of urine marking events by gray wolves in the absence of chemicals (N = 5) to those in the presence of chemicals (N = 12). The p-values below the lines are associated with comparisons urine-marking events (left value) and scratching, rubbing, digging, scat, and all associated combinations (right value) in the absence (dark bars) and presence (light bars) of chemicals, while the p-value above the line is associated with comparisons of overall changes in scent-marking behaviors in the presence or absence of chemicals.

Discussion

In this study, chemicals isolated from the urine of gray wolves and red wolves were tested to determine their impact on behaviors specifically associated with scent-marking or territory marking. The combination of chemicals found in the CAM (control/acetophenone/methyl-propyl sulfide) combination was the most effective of the chemicals tested in that it produced an observational increase in overall urine-marking by the wolves, especially in the number of raised leg urinations by the males. This is of particular interest for several different reasons.

First, previous mammalian studies show that methyl propyl sulfide is found in greater quantities in males (Raymer, et al., 1984; Mech and Boitani, 2003; Zhang, et al., 2005) while acetophenone (Raymer, et al., 1984) is found to be in greater quantities in females, providing several plausible explanations for the increased RLU events. In reference to chemical methyl-propyl sulfide, it could be that the males are detecting the increase in the chemical along their territory boundary and recognize it as a male dominant chemical. They in turn "re-mark" their boundary to ensure that "intruding males" know the territory is occupied. This is also an explanation for the lack of interest and activity observed at gray wolf study site 3 where the pack consisted of only female animals. Other studies focusing on the reproductive strategies of wolves have shown that methyl-propyl sulfide levels are elevated in male wolves during the breeding season (Raymer, et al., 1984; Mech & Boitani, 2003), which can occur anytime between January and March. Thus, an increase in this chemical in the present study where it was introduced outside of the wolves breeding season, could be sending a sexual message that mimics that of signals released during reproductive periods. It would be interesting to see what the interest level of the wolves would be when introduced to this chemical for a full 12-month study taking into account interest levels in and out of the breeding season.

In the CAM combination treatment, the interest level of the wolves increased considerably with the addition of the acetophenone in conjunction to the methyl-propyl sulfide. One explanation for this could be that the increased amount of

acetophenone, found in greater quantities in female canids, again produced a gender-based response in the males that may correlate with reproductive activity. Additionally, when looking at the increased scent-marking behaviors of the wolves in the presence of the CAM combination, and the number of days between placements of the acetophenone before the addition of the methyl-propyl sulfide seemed to be important. Observations indicate other factors may be influencing the chemical message being received by the wolves such as a degradation of acetophenone over time. This in turn may be producing a new or altered chemical message or a combination of the acetophenone and the methyl propyl sulfide producing a novel third chemical which then conveyed a separate message. A chemical analysis study is currently being conducted to determine if a chemical change is occurring in the CAM combination and is the trigger for scent-marking behaviors.

Finally, studies show that scent-marks are often deposited in locations where previous urine-marks were placed and the regular use of these urine-marked routes (such as the location of the scent station sites) have been shown to produce positive feedback for the animals, increasing the probability of remarking (Peters and Mech, 1975; Nunez and Javier de Miguel, 2004; Miklosi, 2008). In addition, frequent exposure to familiar odorants, such as urine-marked boundaries, can also create learned patterns and establishes recognition of territory as well as pack members (Wilson and Stevenson, 2006; Miklosi, 2008). All of which implies that the CAM combination may be triggering the wolves response to reaffirm social status and the familiarity of the territory for themselves as well as pack mates. Exploitation of this basic ecology could prove useful in the manipulation of territory boundaries. The results from the current study indicate that the CAM chemical combination is producing a semio-chemical message that appears to be causing the wolves to re-mark their territory. If this is in fact the case, the implications for this could be further expanded to assist wildlife managers in the development of bio-boundaries to assist with problematic wildlife. If the wolves recognize a chemosensory territory boundary line, and the marks (chemical placements) are fresh, the wolves may assume a territory is occupied. This in turn may modify

behavior preventing the animal from crossing into the space for fear of encountering the inhabitants, thus preventing potential livestock loss. Several studies are currently being conducted to determine the effectiveness of such bio-fences in both African wild dogs (Apps, 2012) and in wolves (Ausband, 2010) and it has been determined that human-marked boundaries may in fact modify behavior (Ausband, 2011), but additional studies are needed. The study involving the wolf bio-fence has incorporated the use of complete urine and scat which can be often hard to procure and may be costly for potential users outside of the scientific community. By identifying specific chemicals in urinary profiles that could be manufactured synthetically, wildlife managers and others could effectively cut costs and ensure that farmers and ranchers can re-mark boundary lines as needed by simply ordering the needed chemical. This further demonstrates the importance of studies identifying specific chemicals or chemical compounds that influence animal behaviors.

Additionally, it is important to note that the use of urine as a positive control would have been beneficial as it would have provided verification that behavioral responses to the respective synthetic chemicals had some relevance to natural urine samples. As restrictions were placed on the study by captive facilities due to the potential transmission of zoonotic diseases, urine as a positive control was prohibited. Further expansion of this study in a field setting, observing the behaviors of wild wolves where urine could be used as a positive control would be of great interest.

Overall, the current study provides a greater understanding of specific chemical compounds found in the urinary profiles of canids and their impact on scent-marking behaviors in wolves. As the majority of studies on the chemosensory signals in wolves have focused on reproduction (Anisko, 1976; Asa et al., 1990; Raymer, et al., 1984; Mech and Boitani, 2003; Packard, 2003), and the results presented here indicate that several of the chemicals associated with reproduction are also triggering scent-marking behaviors, perhaps one of the best management strategies would be to exploit sex as a means of manipulating behavior in

problematic wildlife. Furthermore, the behavioral responses in this study may also be linked not just too reproductive behaviors, but also to territory maintenance. The chemical combination (CAM) identified by the current investigation may prove a useful tool in the ongoing search for non-lethal predation control measures such as the manipulation of perceived territory boundaries and the use of bio-fences. Using the basic chemical compounds found in canid urinary profiles is more cost effective and easier to produce synthetically then collecting scat and urine samples from other animals. The chemicals identified here could be further explored and potentially used to development tools for the manipulation of wolf behavior.

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Chapter Five

Chemical Air Analysis of Volatile Organic Breakdowns Associated with Scent-Marking Behaviors Seen in Canids

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Abstract

The isolation of individual odors is a complex function often influenced by environmental conditions and the natural process of chemical degradation over time. The result of these processes, often change and impact the behavioral responses of the animals that encounter these signals. In a recent behavioral study on the urine-marking of canids, it was noted that aged acetophenone when combined with methyl n-propyl sulfide caused animals to urine- mark. Here we replicate the aging process of acetophenone in a controlled environment, while monitoring any changes over time prior to adding methyl n-propyl sulfide to the aged acetophenone. We then sampled the combined volatile compounds in order to identify possible chemicals associated with urine-marking events. Several compounds were identified that occur naturally in urinary profiles of canids to include propanal, acetic acid, 1-butanol, and naphthalene. We found certain species-specific chemicals, some of which were not associated with the original urinary profiles of the canids, indicating that the original chemicals may degrade to produce new chemicals or chemicals that mimic the chemosensory signals transmitted by secondary species, and these apparently impact behavior.

Key words: Acetophenone, air samples, chemical signals, methyl propyl sulfide, urine, scent-marking,

Introduction

Animals release a myriad of chemicals into the environment as either deliberate chemosensory signals providing information on gender, reproductive status, or as bi-products of metabolic processes and waste excretion (Childs-Sanford 2005; Sanchez-Andrade and Kendrick 2009; Touhara and Vosshall 2009). These chemical molecules can convey information independently or as a more complex chemical mixture to elicit specific behavioral responses (Doty 1986). For example, 2-methylbut-2-enal (2MB2), also called the "nipple pheromone" in rabbits, triggers specific behavioral responses in pups to facilitate nursing behavior (Schaal et. al. 2003; Brennan and Keverne 2004) and acts as a single independent chemosensory signal, whereas methanothiol (MTMT), present in the urine of male mice as a female attractant (Brennan and Keverne 2004; Sanchez-Andrade and Kendrick 2009), is a more complex mixtures of molecules. Both 2MB2 and MTMT are examples of odorants, chemical that can be detected by an animal's olfactory system (Conover 2007; Sanchez-Andrade and Kendrick 2009; Touhara and Vosshall 2009).

Most important odorants are volatile organic compounds with molecular weights lower then 300, and it is estimated that the number of detectable odorants ranges in the hundreds of thousands (Conover 2007; Touhara and Vosshall 2009). Animals are constantly releasing different odorants, which become a mass of chemosensory signals in the environment. However, animals like olfactory predators, which hunt primarily using smell, have developed the ability to sort through the array of environmentally present odorants to find specific chemicals associated with their prey (Conover 2007). In fact, one group of renowned olfactory predators, the canids, has long been recognized for its ability to ignore the "background matrix" of chemical cocktails and isolate single odors (Harper, et.al. 2005).

Isolation of individual odors is a complex procedure and is often influenced by the environment. Environmental factors such as temperature, wind, humidity, and barometric pressure, can cause the chemical compounds to breakdown over time.

Breakdown ensures a conveyance of an ever changing array of information, impacting and changing animal response behaviors to the odorants. One of the most familiar forms of chemical communication is that of urine-marking territory by animals. When marking territory, animals often select a variety of substrates depending on the method of marking to ensure the presence of both visual and olfactory cues, and to compensate for environmental impact on the breakdown of the chemical messages being left. Canids, such as wolves for example, often use urine, scat, and scratching to identify territory boundaries. Wolves will also mark elevated substrates such as trees and other vegetation (Peters and Mech 1975; Nunez and Javier de Miguel 2004) to ensure the long slow diffusion of a chemical and thereby increasing its effectiveness as a signal (Donovan 1969), while also compensating for the natural chemical degradation process over time.

As a result of this diffusion process into the environment, and the slowing of chemical evaporation rates, most odorants are found in mixtures, such as urine, typically made up of many small molecules of molecular weight < 300 (Conover 2007). Because these odors are usually a matrix of compounds and are directly impacted by environmental conditions, it is difficult to determine exactly which compound in a mix is acting as a signal. For example, sometimes, a single odor is detected, other times the combination of two odors creates a third new odor, while some odors mask the presence of other odors or even strengthen or weaken a single odor when the chemicals are placed together (Conover 2007).

Several studies have been conducted using air samples of scent marks (Soini et. al. 2005; Childs-Sanford 2005), but none have focused on the natural breakdown of the chemicals over time. In a recent study involving organic chemicals isolated in a urinary profile of gray wolves (Chapter 2), several chemicals were studied to determine their impact on behavior (Chapter 3). It was noted that urine-marking behavior was induced when methyl n-propyl sulfide was added to aged acetophenone. Based on this finding, it appears that the natural breakdown of the chemicals may trigger urinary marking by wolves. This raises a variety of questions: is the acetophenone degrading and was the combination of organics

producing a chemical signal that triggered the dominant animals to urine-mark, or was it something else? For example, the aged acetophenone combined with un-aged methyl n-propyl sulfide could be producing a chemical compound found previously in urinary profiles of the canids, or it could be creating a new compound unidentified in the urinary profiles, which would warrant further behavioral testing. In any case, some interaction is occurring between these two chemicals that generates a behavioral response. Here, we replicated the field experiment in a laboratory environment in order to analyze the chemical breakdown of the organics over time to determine if they are in fact degrading, and if so what possible combinations may be responsible for the urine-marking behavior seen in the behavioral study.

Materials and methods

Chamber Setup

In order to collect volatile compound samples triggering urination in wolves in their natural habitats a volatile organic compound (VOC) chamber at RTI International was used. The chamber consisted of a borosilicate glass tube (36cm L x 6cm diameter x 0.6cm wall thickness) tooled with an O-ring groove to provide a tight seal when joined with end-joints (Figure 1). Pinch clamps were used to secure the chamber body to the end-joints with one end-joint attached to the sampling tubes and the second connected to the house nitrogen supply through a stainless steel tube (Figure 2). Prior to the running experimental samples, the outside of the chamber was wrapped in heating coil and glass wool insulation materials and heated for 72 hours at a coil temperature of 100°C and under nitrogen flow (150mL/min) to ensure any residual compounds had volatized and been swept out of the chamber.

ATD Cold Trap and Sample Tube Conditioning

The ATD cold trap was monitored and deemed free of residual contamination by directing the helium flow through the GC/MSD for 1-hour at a temperature of 350°C.

Figure 1. Glass Chamber with Tooled O-ring Grooves

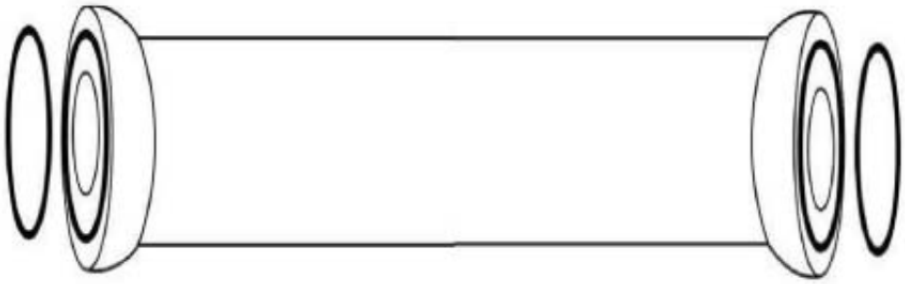


Figure 2. VOC Chamber at RTI Laboratories

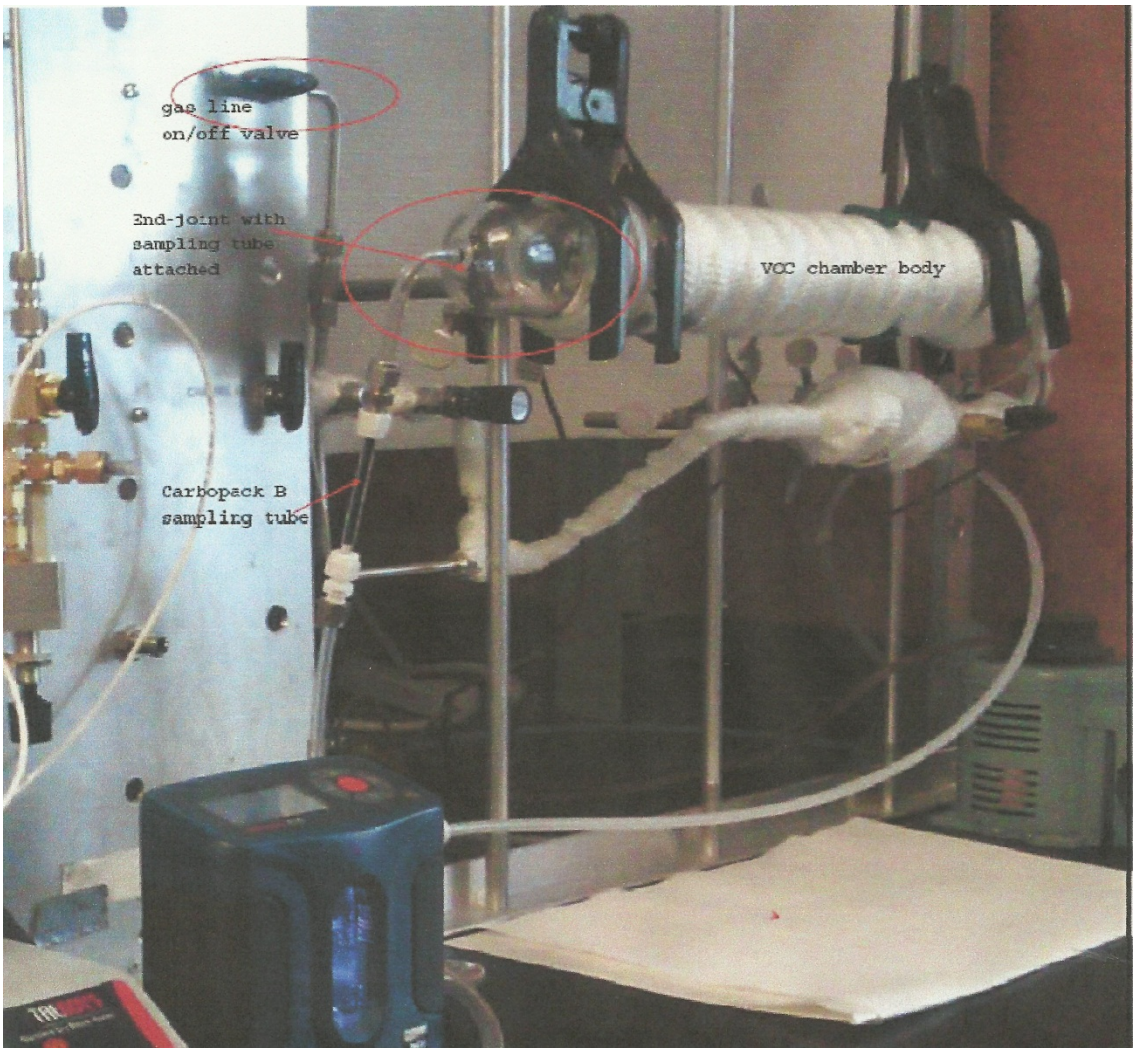
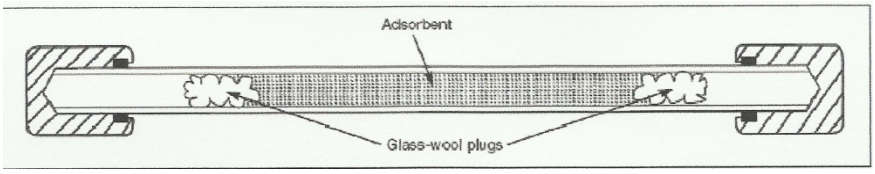


Figure 3. Active Sampling Tube



Pre-packed, glass sampling tubes containing the adsorbent Carbopack™B 60/80 were conditioned on the ATD as instructed by the manufacturer. The sample tubes were heated under the flow of helium (75 mL/min) for 30min at 350°C allowed to cool and capped with Teflon end caps (Figure 3).

Sample Preparation and Loading

Sample Blank

One sample blank was analyzed daily before each target sample below. The sample blank consisted of a conditioned Carbopack™ B tube not loaded with a target compound analyzed for determination of residual compounds. The same tube, after analysis, was then loaded with effluent from the chamber containing the target compound and analyzed as a sample as detailed below.

Sampling Acetophenone

In order to duplicate the field experiment internally, we prepared the samples using the same 12ml Nalgene vials with snap cap lids containing a single hole that were used in the field study and placed a sterile cotton ball inside. Using a 1mL Hamilton gas-tight syringe, we spiked the cotton ball through the hole with 1mL of Acetophenone. The VOC chamber which contained a BIOS Defender 510 flow meter (SKC) with a Nitrogen flow of approximately 70mL/min was opened; the vial was placed immediately inside and then resealed with the nitrogen being vented to the hood until sample collection (Figure 2). Acetophenone samples were allowed to equilibrate in the chamber for 6 hours prior to the Day 1 sample collection. During the sample collection, the ventilation tube was removed from the end-joint and replaced with a Carbopack™B 60/80 tube. A flow of 100mL/min was established through the sample tube for 10min at room temperature (23°C). We collected approximately 1000mL of sample. The sample tube was then removed and the ends secured with a Teflon end cap. Two samples were collected for Day 1 and analyzed immediately following sampling. The procedure was repeated for sample tubes collected on Day 3 and Day 4 of the experiment.

Sampling Methyl n-Propyl Sulfide (MPS) on Day 4

After we collected the single Day 4 sample of acetophenone, a fresh cotton ball was placed into a second 12ml Nalgene vial with a snap cap lid containing a single hole. The same as in the Acetophenone sample preparation. Using a 1mL gas-tight syringe, we spiked the cotton ball through the hole with 1mL of methyl n-propyl sulfide. The VOC chamber was opened, the vial placed immediately inside next to the aged acetophenone vial. The chamber was closed and the compounds were allowed to equilibrate for several hours prior to sampling. The effluent of the chamber (1L) was sampled using a Carbopack tube in the same manner as the acetophenone-only samples.

Carbopack Sample Analysis

Immediately following sample collection, the Carbopack tubes were placed onto the ATD carousel and desorbed at 300°C under a flow of helium for 15 minutes. The effluent was trapped at -30°C on a secondary trap and was introduced to the GC by heating the trap at 40°C/sec to 325°C and were swept to the head of the column (J&W DB-5ms, 50m x 250µm x 0.25µm) at 10 mL/min via a heated transfer line (250°C). Following data acquisition, the files were then processed for peak identification using the NIST library spectral matching. The GC-MS analysis parameters are represented in Table 1.

Despite the conditioning of the Carbopack™ B tubes several times, some contaminants were observed within the sample blanks, likely from previous use, and are shown in Table 2. Compounds found consistent with the aged acetophenone, the aged acetophenone and methyl propyl sulfide combination, and the blanks themselves were removed when looking at associations between identified compounds.

Table 1. GC / MS Parameters

Parameter	Setting
Oven	
Initial Temperature	70°C
Initial Time	5 min
Rate	8°C min^{-1}
Final Temperature	275°C
Final Time	16.37 min
Total Run Time	47 min
Injector – External device [ATD] Column	
Head Pressure	10 psi
Flow	10.0 mL min^{-1}
Mass Spectrometer	
Transfer Line Temperature	250°C
Mass Scan Range [full scan]	30-550amu
Electron Impact [EI]	70eV
Scan Rate	1.44 scan/sec

Table 2. Sample Blank Compounds – Contaminants

COMPOUND	**RETENTION TIME**	**NIST MATCH**
Acetone	4.22	40
(Z)-1-chloro-1-Buten-3-yne	4.34	45
Pentanal	4.56	42
Benzene	5.074	96
2,2,4-Trimethylpentane	5.287	83
2,2,3,3-Tetramethylbutane	5.372	83
Toluene d8	6.484	91
Hexamethylcyclotrisiloxane	7.142	83
Diphenylamine	24.891	83
Dibutyl phthalate	29.568	95

Results

Compound Identification

All compounds were processed for peak identification using the NIST library spectral matching with a spectral match ranging from ≥35% to ≤100%. Aging acetophenone compounds are detailed in Table 3 and aged acetophenone and methyl propyl sulfide (MPS) compounds are detailed in Table 4.

In analyzing the compounds generated by aging acetophenone over 4 days we find that there were a total of seven compounds specific to day one. These may represent the initial chemical breakdown of the compound or possibly imperfections in the acetophenone. By day three, only one day-specific novel compound, 5-Benzolypentanoic acid, was identified. A single compound 2, 2-dimethylhexane, was found on both day one and day three and four compounds were found consistently across all days. Acetic acid, Octamethylcyclotetrasiloxane, 1-(2-methyl-1-cyclopentenyl) ethanone, and pure acetophenone were found across all four days, but there was variation in the quantity of these compounds across sampling days. For example, we see a decrease in the amount of 1-(2-methyl-1-cyclopentenyl) ethanone in the overall air sample from day 1 through day four. Likewise, we see a decrease in the amount of cyclotetrasiloxane and the amount of acetic acid from day one through day four (Figure 3). However, we did see an increase in the amount of acetophenone from day one through day four indicating a purification of the acetophenone in the air sample (Figure 4).

In analyzing the compounds from the combination of the aged acetophenone combined with the methyl propyl sulfide, we find that there were a total of thirteen identified compounds. Compounds not identified in the aged acetophenone may represent the initial chemical breakdown of the methyl propyl sulfide, possible

Table 3. Aging Acetophenone Chemical Compound Identification

Day 1 – Specific	**Retention Time**	**NIST Match**
Hexane	4.564	47
1-Butanol	4.991	78
2,3-Dimethyl-2-pentene	6.971	59
4-Methylmorpholine	13.205	38
Naphthalene	16.947	95
Butyl Octyl Phthalate	28.783	53
9,10-Dihydro-9,9-dimethylacridine	29.452	91
Day 3 - Specific		
5-Benzoylpentanoic acid	16.96	43
Day 1 / Day 3 - Specific		
2,2-Dimethylhexane	5.368	83
Day 1 / Day 3 / Day 4 -Specific		
Acetic acid	4.381	90
Octamethylcyclotetrasiloxane	11.231	83
1-(2-methyl-1-cyclopentenyl)ethanone	13.673	58
Acetophenone	14.057	95

Figure 4. Area percentage in the air column of select compounds identified in all three sample days 1, 3, and 4 from the aging Acetophone.

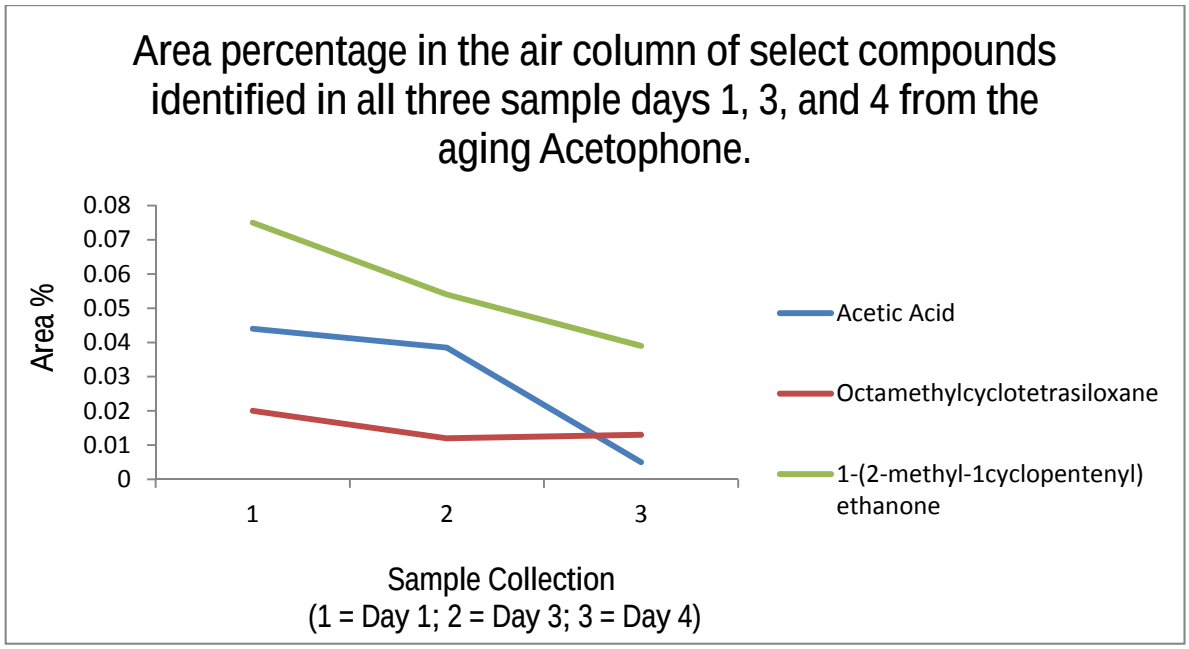


Figure 5. Area percentage in the air column of Acetophenone identified in all three sample days 1, 3, and 4 from the aging Acetophone.

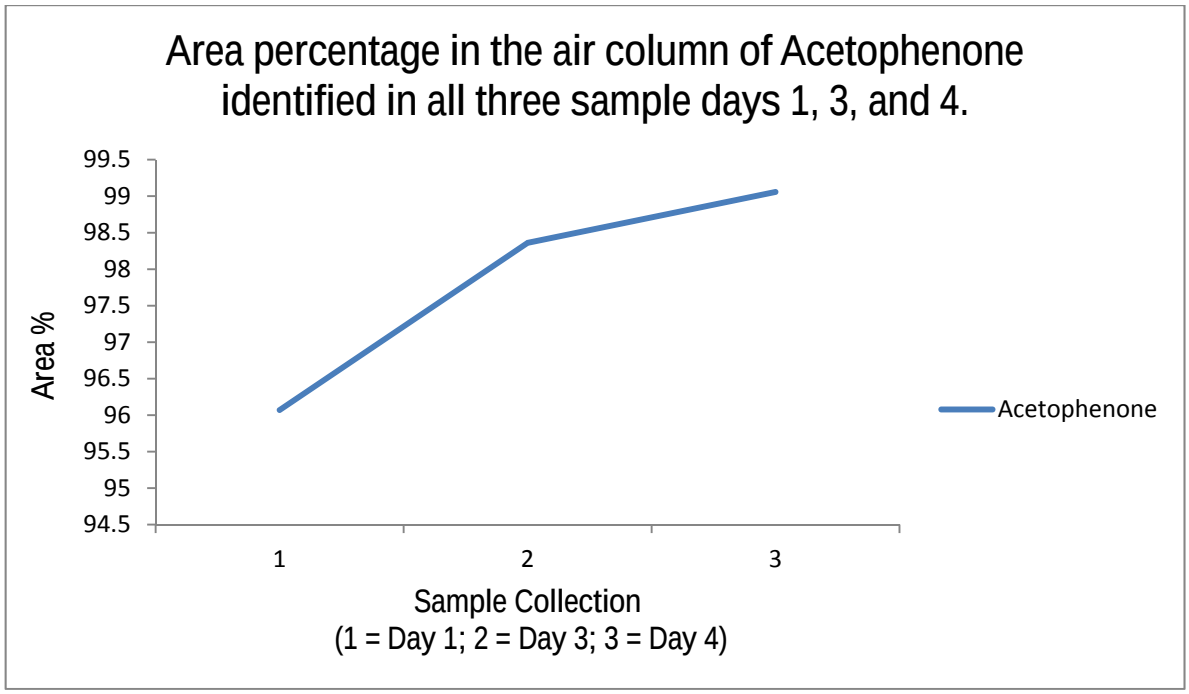


Table 4. Aged Acetophenone and Methyl Propyl Sulfide (MSP) Chemical Compound Identification

Compound	Retention Time	NIST Match
Propanal	4.279	53
Formic acid, propyl ester	4.441	42
Butanal	4.623	86
1-propanethiol	4.75	94
N-Propylbromide	4.869	72
2-Bromobutane	5.449	86
Methyl n-propyl sulfide	5.988	95
Dimethyl disulphide	6.194	98
Sec-butyl Methyl Sulfide	6.706	74
Propyl Sulfide	9.242	95
Thiazole, tetrahydro-	9.522	40
1-(2-methyl-1-cyclopentenyl)ethanone	13.756	35
Acetophenone	14.103	94

imperfections in the methyl propyl sulfide or actual chemical combinations as the aged acetophenone is introduced to the methyl propyl sulfide. The compounds associated specifically with these possibilities include propanal; formic acid, propyl ester; butanal; 1-propanethiol; n-Propylbromide; 2-Bromobutane; dimethyl disulfide; Sec-butyl Methyl Sulfide; Propyl Sulfide; and thiazole, tetrahydro-.

Discussion

Studies of various mammal species indicate that acetophenone is found in greater quantities in females (Jorgenson et al., 1978; Raymer, Holland et al., 1984; Zhang et al., 2005) while methyl propyl sulfide is found specifically in males (Raymer, Holland et al., 1984). These two compounds are also present in the urinary profiles of canids and incite a strong behavioral scent-marking response (Chapter 4). As a result, both acetophenone and methyl propyl sulfide could be classified as odorants. This is because they trigger a behavioral response in an animal that relies heavily on its sense of olfaction and their molecular weight (the molecular weight of acetophenone is 120.15 g/mol^{-1} while the molecular weight of methyl propyl sulfide is 90.19 g/mol^{-1}) is consistent with that of odorants. Molecular weight is directly correlated to the length of time the chemicals remain in the air, with heavier molecules dropping from air streams faster, preventing their detection over longer periods of time (Conover 2007). As a result, we expected to see a drop in the amount of pure acetophenone suspended in the air samples from day 1 to day 4, possibly impacting the chemosensory signals detected by the canids in the behavioral study. However, we found the opposite, with more acetophenone suspended in the air matrix over time representing a possible amplification of the compound detected by the animals in the field study.

Originally the experiment was designed to use PDMS stir bars to recover volatile compounds from a closed system and to look at the acetophenone aging process day 1-day 4; un-aged acetophenone and methyl propyl sulfide independently, and finally to analyze the mixture of the aged acetophenone and the methyl propyl sulfide. However, due to limited funds, the experiment was modified to reflect only the analysis of aging acetophenone and a mixture of aged acetophenone with the

methyl propyl sulfide using the analytical technique specified in the methods section.

It is nonetheless clear that the chemical changes occurring over the sampling period are small and include only twelve volatiles from the aging acetophenone and thirteen from the acetophenone/methyl propyl sulfide mix. When comparing the compounds from this study to the chemical breakdowns of the urinary profiles for the male / female gray wolf (Raymer 1984; Chapter 3), the domestic dog (Raymer 1984; Chapter 3), the wolf-dog hybrid (Chapter 3), and the male / female red wolf (Chapter 3), a few similarities were noted. The chemical compounds common across studies include, propanal, found in the urinary profile of the domestic dog (Raymer 1984), acetic acid, found in all 4 canid species (Chapter 3), 1-Butanol, found in the urinary profile of the gray wolf male and female (Chapter 3), and naphthalene found in the domestic dog (Raymer 1984). These compounds, identified in both the air samples and in the urinary profiles, require further behavioral studies to assess whether or not they are behavioral triggers of any description. There relative consistency suggest they may well be important signals.

The current study raises many questions. Firstly, is the chemical degradation producing a compound associated with the presence of a secondary species triggering scent-marking behaviors in the wolves? In other words, is the degradation producing a chemical not associated with wolf urinary profiles but instead one found in the urinary profiles of the domestic dog (propanal and naphthalene)? Secondly, are the chemicals triggering detection of a single odor or a compound mixture where any of the compounds from Table 3 are inciting a behavioral response? Thirdly, are the chemicals identified in Table 3 creating a novel odor that triggers a scent-marking response? Or finally, could the primary chemicals acetophenone and methyl propyl sulfide be strengthening/weakening one another when placed together inciting the noted behavioral response? One possible indicator supporting this last possibility is that we saw an increase in the purity of the acetophenone as it aged from day one to day four increasing from

97.604% of the air sample to 99.059% of the air sample. The purification may then produce an amplification of the compound triggering the urine-marking event.

Overall, the study identified chemical compounds associated with aging of acetophenone over a four day period and the identification of the chemical compounds associated with the mixture of aged acetophenone and methyl propyl sulfide. Further behavioral studies of all the compounds found in this study are needed in or order to clarify exactly what role, if any, the mixture of the aged acetophenone and un-aged methyl propyl sulfide along with the specific chemical compounds identified have on scent-marking behavior. In addition, the blending of the compounds in future behavioral analysis along with doses more appropriate to quantities found in actual urine would prove beneficial and would also further enhance the identification of mixtures leading to scent-marking behavior.

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Chapter Six

General Discussion:

Scent-Marking: Investigating Chemosensory Signals in Wolf Urine

There has been human-wolf conflict for centuries. These large carnivores have the broadest natural distribution of all land-based mammal species aside from humans (Musiani & Paquet, 2004) and as a result, they come into contact and compete with humans for certain resources, including livestock. Livestock depredation is one of the main sources of contention fueling the human-wolf conflict and has been one of the great concerns as current public opinions have shifted to biodiversity preservation. Many livestock owners fight to maintain their rights to use lethal control measures to alleviate problematic wildlife, while conservationists fight for the use of non-lethal control measures. Wildlife biologists often find themselves in the middle of these disputes and bridging the gap to find a mutual balance that benefits the animals as well as humans is somewhat challenging.

This thesis sought to address part of this challenge and see if a method or tool could be provided to benefit biologists in the development of more effective strategies to reduce depredation through non-lethal management tools. In order to accomplish this, the study looked to exploit basic wolf biology. Wolves are highly social pack animals usually led by a dominant male and female. Animals hunt as a social unit and maintain territories. These territories usually remain stable and exclusive over time (Taylor & Pekins, 1991), with territory size being determined by resource availability. Wolves will travel their territories in irregular patterns and will reach all boundaries about every three weeks (Peters & Mech, 1975). During the duration of travel, the dominant wolves are re-marking territory boundaries using a variety of scent-marking methods to include raised-leg urinations (RLU), squat-urinations (SQU), defecation, and scratching (Asa, et al., 1985; Peters & Mech, 1975; Zub, et al., 2003). It has been suggested that foreign scent marks incite an avoidance response in wolves (Briscoe, et al., 2002; Paquet, 1991; Peters & Mech, 1975) in turn creating a behavioral aversion response as a result of unfamiliar scent marks (Peters & Mech, 1975). Therefore, if the effects of foreign scent marks could be duplicated synthetically through the identification of the chemosensory signals triggers and used as a behavioral modification tool to reduce predation, the basic biology of scent-marking becomes of particular interest.

In previous studies investigating aspects of basic biology, such as chemosensory signaling, the work has focused primarily on reproductive strategies. In this thesis, it is believed that these signals, whether associated with reproduction or simple territory maintenance, could be identified and then used to develop management tools for problematic wildlife. The concept of bio-fences is not new, yet very little research has been undertaken to try and identify compounds associated with the territory maintenance of large carnivores. My study looked at the basic biology of chemosensory signals in the urine of canids and the influence of various organic compounds on scent-marking behavior. The thesis was designed to accomplish several goals.

The first goal was to develop a urinary profile for each canid species in the study by using less invasive sample collection, modern technology, and more advanced extraction methods (Chapter 2 and Chapter 3). As non-invasive collection procedures have become more prominent in the field of wildlife biology, the use of new analytical technologies and sample preparation techniques are critical to the analysis of these types of biological samples. These less invasive techniques often garner small quantity or dilute samples making it harder or even impossible to extract volatile organics using older extraction methods, which typically require larger, more pure samples. In using more advanced instrumentation, such as a current gas chromatograph (GC) that can now theoretically separate over 300 solutes in a single run (David, et al., 2007), along with the increased sensitivity of the mass spectrometer (MS), and extraction methods such as the stir-bar sorptive extraction (SBSE), the study was able to effectively detect and identify a total of one hundred and forty four compounds in dilute male urine samples and one hundred and two compounds in dilute female urine samples across four canid species, the gray wolf (*Canis lupus*), red wolf (*Canis rufus*), wolf-dog hybrid (*Canis domesticus*), and the domestic dog (*Canis domesticus*).

The second goal in the thesis was to compare the canid urinary profiles across all species and genders to determine similarities and differences (Chapter 3), and to isolate compounds found in all four canid species for behavioral testing. The results showed that no two canid species or genders contained exactly the same

chemical compounds in their urinary profiles. Although several of the compounds were the same (Chapter 3: Table 4; Table 6), many of the compounds were distinct among species (Chapter 3: Table 2; Table 5) and among genders (Chapter 3: Table 8; Table 9), signifying that field biologists could now identify gender and species of certain canids strictly from urine samples. In addition, gender differences both inter- and intra-specifically can be used for future reproductive studies and may provide helpful information for various management programs especially those associated with the red wolf and the current issues involving the breeding of red wolf females with coyote males.

Comparisons of the canid urinary profiles with the chemical compounds associated with urine-marking in other carnivores, such as tigers (Burger, et al., 2008), lions (Andersen & Vulpius, 1999), fox (Jorgenson, et al., 1978), ferrets (Zhang J. , et al., 2005), and wolverines (Wood, et al., 2009) provided additional information in the identification of certain compounds thought to be associated with urine-marking behavioral patterns. This was important to this study as I was looking to identify compounds found across the four canid species that are associated with urine-marking, in order to identify the compound or compound mixes most likely to be responsible for triggering urine-marking. In comparing the results from the canid urinary profiles to those of other carnivores, I identified several compounds common across carnivore species that do trigger urine-marking and investigated these compounds in the wolves.

The first and second goals were successful in that six species and gender specific urinary profiles (Appendix I) were recorded and multiple similarities and differences were found. The resulting urinary profiles provided reliable data that could subsequently be used for identifying chemosensory signals involved in reproduction, territory maintenance, scent-marking behaviors, and even predator/prey dynamics. Two of the urinary profiles to include both the red wolf and the wolf-dog hybrid (Chapter 3) provided information on two canids which had not been previously analyzed. In addition, compounds in all four canid species (Table 1) and across male carnivores (Table 1) were identified to be tested in behavioral studies to determine which if any triggers urine-marking events.

TABLE 1. Organic compounds found across all four canid species and genders

COMPOUND	COMPOUND
acetic acid*	2-pentanone
2-heptanone*	4-methyl-2-heptanone
Acetophenone*	Nonanal*
Benzene, 1,3-bis (1,1-dimethylethyl)	nonanoic acid*
Quinoline, 2-methyl*	8-Quinolinol, 2-methyl
phenol, 2,4-bis(1,1-dimethylethyl)	tetradecanoic acid*
Z-11-hexadecenoic acid	N-Hexadecanoic acid*
2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide	9,12-Octedecadienoic acid (Z,Z)-
(E)-9-octadecenoic acid	Octadecanoic acid

Note: (*) indicates compounds found in the urinary profiles of other carnivores thought to be associated with urine-marking events.

The third goal of the thesis was to test if the chemical compounds common across species and implicated in scent marking in other taxa had any effect on the behavior of both the gray wolves and the red wolves (Chapter 4). The study focused on finding out if any of these chemical compounds triggered scent-marking behaviors (Chapter 4).

The results showed that captive wolves (red wolves and gray wolves) increased activity level when chemicals were placed at the scent-stations along territory boundary lines. The chemical combination with the greatest influence was the CAM (control/acetophenone/methyl-propyl sulfide) treatment which triggered increased urine-marking. The increase in urine-marking was primarily recorded through the number of raised-leg urinations (RLU) by the dominant males in the presence of the CAM treatment and there are several plausible explanations for this: 1) methyl-propyl sulfide (MSP) is found in greater quantities in males and acetophenone is found in greater quantities in females, thus the combination of the

two increases the effect of the MSP causing the males to recognize it as a male dominant chemical stimulating “re-marking” their territory; or 2) previous studies on reproductive strategies of wolves have linked the increase of MSP in males to the breeding season (January – March) (Raymer, et al., 1984; Mech & Boitani, 2003), thus in introducing the chemical outside of the breeding season, the study sent sexual messages mimicking signals released during reproductive periods. In addition, if the signals are in fact mimicking sexual cues, the question posed then becomes are the signals acting as a deterrent or an attractant and is this signal dependent upon reproductive seasonality? Future studies addressing this would be most beneficial and would provide further insight into the use of sex as a means to deter problematic wildlife. Either way, a message was being received by the wolves that triggered an increase in scent-marking behaviors.

The third goal was therefore also successful. However, further study is needed in order to determine what signal is actually being imparted in the chemical message by the CAM treatment, whether reproductive or a territory maintenance response. Regardless, the chemicals could be used to establish faux territory boundaries sending messages to other wolves that the area is occupied by a new bonded pair. As a result, the chemicals could then be used to assist in developing non-lethal control methods such as bio-fences to aid in deterring predation. Further studies would need to be conducted in a wild setting opposed to a captive setting in order to determine similar results.

The final goal of the thesis was to see if the aging or breaking-down of the compounds was creating a new compound(s) that was triggering scent-marking behaviors (Chapter 5). It is known that canids re-mark territory boundaries on a regular basis (Asa, et al., 1985; Peters & Mech, 1975; Sillero-Zubiri & Macdonald, 1998) and will overmark urine-marks by other animals (Wyatt, 2003). Therefore, the compounds that triggered the scent-marking behaviors (Chapter 4) may not be directly or independently responsible, but may be producing another novel compound that triggers the event. In order to test this, the study had to identify several possibilities for the urine-marking behavior: 1) is the acetophenone

degrading and if so into what; 2) are the degradation compounds from the acetophenone when combined with the methyl-propyl sulfide triggering the urine-marking event or is the combination of the acetophenone and the methyl-propyl sulfide mix creating another compound not related to the degradation of the acetophenone; and 3) are any of these new compounds from either the degradation of acetophenone or the combination of aged acetophenone and methyl-propyl sulfide found as existing chemicals in the urinary profiles.

What the study found was that the acetophenone did have additional chemical compounds associated with it but that the chemical itself only appeared to become more pure over time. The air sample containing the combination of the degrading acetophenone and the methyl propyl sulfide showed to have thirteen different chemicals in addition to the two being tested and several of the compounds associated with the degradation of the acetophenone and the aged acetophenone and methyl-propyl sulfide mix were found in the urinary profiles indicating that they could be responsible for triggering urine-marking behavior. Further behavioral studies would be needed in order to determine if they had a significant impact on urine-marking behavior.

Despite, the alterations to the original lab work for this section of the thesis due to budgetary limitations, a definitive answer was not found to the question regarding the identification of the specific compound triggering urine-marking behaviors. However, the work in this section of the thesis does lay the foundation for additional research to further ascertain which compound or compound mix may be responsible for directly triggering urine-marking behaviors.

Overall, the thesis identified organic compounds in six urinary profiles for four canid species (two of which had never been identified), compared similarities and differences across genders and species, identified both acetophenone and methyl-propyl sulfide as being possible triggers for urine-marking events, and analyzed air samples to determine if the aging of the chemicals were creating new chemical

compounds triggering urine-marking behaviors. The implications of these findings are numerous.

First, the thesis illustrates the benefits of technological advances and shows how an extraction method, such as SBSE, can enhance the identification of compounds in dilute aqueous matrices. In previous studies using other types of extraction methods, biologists had to acquire large quantities of sample requiring invasive collection techniques, such as collecting urine from immobilized animals (Raymer, 1984; Asa, et al., 1985), in order to obtain useful samples for bioanalysis. This type of collection can limit sample availablity making it harder to acquire sample quantities required for analysis. In this study, dilute or small quantity urine samples along with samples collected using non-invasive collection methods (urine samples collected from snow) are shown to be just as effective for bio-analysis using the SBSE method. The use of new extraction methods, such as SBSE, coupled with more sophisticated instrumentation and digital spectral libraries (which enhance the identification process of sample compounds), removes the restrictiveness of sample quantity when dealing with aqueous matrices, such as urine, and makes sample acquisition easier and more cost effective for biologists who no longer have to immobilize the animal. Other studies using the SBSE method, to include the identification of organic pollutants in water (Tienpont, et al., 2002; David, et al., 2003), profiling of food flavors (Tienpont, et al., 2002; David, et al., 2003), and urine studies in ferrets (Zhang J. , et al., 2005) and mice (Soini, et al., 2005), have also shown the effectiveness of the SBSE method when analyzing dilute samples in samples in small quantities. By using this extraction method to analyze dilute samples of canid urine; this study increased the number of organic chemical compounds identified in the samples due to the SBSE's ability to detect trace amounts of organic chemical compounds which may have been missed using previous extraction methods. As a result, the thesis provided detailed urinary profiles of organic chemical compounds that can aid in the identification of chemosensory signals that modify canid behavior whether reproductive, territory maintenance, and management of problematic wildlife or predator/prey dynamics.

Secondly, the thesis identified chemicals that have the potential to modify canid behavior which can be further investigated for use as a means of non-lethal control for problematic wildlife. The CAM treatment identified in this study influencing scent-marking behaviors in wolves could provide a potential method of non-lethal control through the creation of faux territory boundaries for the management of livestock predation. Through the identification of these olfactory messages from chemicals found in urinary profiles, triggering specific behaviors such as scent-marking, biologist can analyze combinations in a field setting to determine if these chemical cues deter conspecifics thus supporting the idea of a biofence. Similar studies are currently being conducted on African wild dogs (Apps, 2012) and wild wolves (Ausband, 2010) to determine the effectiveness of these faux boundaries or bio-fences. The human-marked boundaries have shown to modify wolf behavior using urine and scat obtained from wolves in other territories which is often hard for the general population to acquire. The results in this study show that chemosensory signals or messages relayed by urine-marks may be chemical compound specific and can be manipulated and even produced synthetically to modify behavior providing easier acquisition and a more cost effective approach to non-lethal predator deterrents such as the biofence. Future studies using these chemicals in settings with wild populations and adding whole urine as a positive control, would provide additional information on their implications as a deterrent further supporting the potential use in biofence research. Additionally, future studies looking at the impact of these chemicals as behavioral modifications associated with the exploitation of sex are also needed to ensure that the signals are in fact deterring conspecifics and not attracting them. If it is found to be an attractant then the chemicals would not be useful to non-lethal management practices through the development of the biofence concept. However, they could be used instead as a non-lethal approach to regulating sexual behaviors and breeding strategies by attracting individuals to areas away from livestock.

Although a specific solitary compound associated with triggering scent-marking behaviors was not identified, the result's in this thesis clearly show that some chemicals such as the CAM combination do impact behavior indicating that using

the basic biology of an animal, such as the chemosensory signals in urine, proves to be beneficial and opens doors to further research opportunities. Furthermore, the results produced this thesis can certainly aid biologists in several ways, first by assisting in the development of new non-lethal management strategies, such as the proposed biofence addressed in the previous paragraph, for problematic wildlife and second by providing useful information for future studies involving reproduction, predator/prey dynamics, territory maintenance and a plethora of other research focusing on animal ecology in association with chemosensory signaling.

In closing, it is arguable to say that most social behaviors in vertebrates are very complicated and are not controlled by a single chemical alone but are instead vastly integrated networks of chemosensory signals, coupled with abiotic factors and other hormonal indicators triggering responses in the brain (Kelliher, 2007). In conducting studies which focus on these components one can better understand the triggers for specific behaviors such as scent-marking and can harness these basic biological events for better management practices.

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Appendix I

Organic Compounds Identified in Male Canid Urine Using SBSE Method

Appendix I – Organic compounds identified in male canid urine using SBSE method

Retention Time [RT]	Compound	CAS #	Gray Wolf	Red Wolf	Wolf-Dog Hybrid	Domestic Dog
1.2679	acetic acid	64-19-7	x	x	x	x
1.5251	1-butanol	71-36-3	x			
1.7351	2-pentanone	107-87-9	x	x	x	x
1.9185	propanoic acid	79-09-4	x	x	x	x
1.9993	methyl propyl sulfide	3877-15-4	x	x	x	
2.3961	3,5-dihydroxybenzamide	3147-62-4			x	
2.3961	methyl isobutyl ketone	108-10-1				x
3.29	acetamide	60-35-5				x
3.5105	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine	1000138-84-6		x		x
3.5105	hexanal	66-25-1			x	
3.5105	methyl cyclohexane	108-87-2	x			
3.9674	n-butyl methyl sulfide	628-29-5	x	x		
4.6884	furfural	98-01-1	x	x	x	x
5.2479	3-methyl-2-hexanone	2550-21-2		x		
5.2479	diacetamide	625-77-4	x			
6.2481	4-heptanone	7379-12-6	x	x	x	x
7.0972	2-heptanone	110-43-0	x	x	x	x
8.372	methoxyphenyloxime	1000222-86-6	x	x	x	
8.8115	α pinene	7785-70-8			x	
9.4022	4-methyl-2-heptanone	6137-06-0	x	x	x	x
9.8428	2,4-diphenyl-2,3-dihydro-1,5-benzothiazepine	4358-31-4		x	X	
9.8428	3,4,5-trimethoxy benzamide	3086-62-2				x
10.143	3-ethylcyclopentanone	10264-55-8				x
10.317	benzaldehyde	100-52-7	x	x	x	x
10.4566	N-(2-methylpropylidene) hydroxylamine	5775-73-5	x			
10.8454	2-nitro- 1,4-benzenedicarboxamide	50739-80-5	x	x		
10.8454	2-formyl-4,6-dimthoxy-,8,8-dimethoxyoct-2-yl benzoate	1000164-89-1			x	
11.5399	benzonitrile	100-47-0				x
11.819	3-octanone	106-68-3	x	x		
12.2782	hexanoic acid	142-62-1			x	
12.2782	pentanoic acid	109-52-4	x	x	x	x
12.2782	2,4,6-trimethylpyridine	108-75-8				x
13.373	benzoxazole	273-53-0			x	
13.373	2-methyl-5-vinyl pyrazine	13925-08-1				x
14.1321	2,2,6-trimethylcyclohexanone	2408-37-9		x		
14.5312	2,4,4-trimethylbut-2-enolide	4182-41-6				x
14.5312	salicylaldehyde	90-02-8	x	x	x	
15.1323	2-methyl-2-hexanol	625-23-0	x			
15.7852	acetophenone	98-86-2	x	x	x	x
16.3448	2-methyl-3-octanone	923-38-4				x
16.3448	1-cyclopropylpentane	2511-91-3	x		x	
16.5213	p-tolualdehyde	104-87-0	x	x	x	x
16.949	3,5-dimethyl-2-octanone	19781-14-7		x		
17.1788	2-nonanone	821-55-6		x	X	
17.1788	heptanoic acid	111-14-8	x		X	x
17.3115	1,2,3,4,5-pentamethylcyclopentene	1000154-28-6	x			
17.9056	nonanal	124-19-6	x	x	x	x
18.3486	2-propylmalonic acid	616-62-6	x			
19.0639	allantoic acid	99-16-1				x
19.8137	succinaldehyde oxime	1000128-11-8			x	
21.4588	benzoic acid	65-85-0	x	x	x	
22.091	hexanoic acid	142-62-1		x		
22.091	octanoic acid	124-07-2			x	
22.9713	decanal	112-31-2		x	x	
23.0635	3,4-dimethyl-2-hexanone	19550-10-8		x		
23.5584	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde			x		

23.756	dihydro-3-pentyl-2(3H)-furanone			x		
23.998	2,3-dihydrobenzofuran	496-16-2			x	
23.998	ethanedithioamide	79-40-3				x
24.2749	imidodicarbonic diamide	108-19-0	x			
24.2749	quinoline	91-22-5		x	x	x
25.132	1,3-bis(1,1-dimethylethyl)-benzene	1014-60-4	x	x	x	x
26.6629	nonanoic acid		x	x	x	x
26.674	(1S-endo)(4,7,7-trimethyl-3 bicyclo [2.2.1] heptanyl) acetate	5655-61-8			x	
27.1463	2-undecanone	112-12-9			x	
27.1463	N-formyl-imidodicarbonic diamide	2148-09-6	x			
27.5535	2-methyl-quinoline	91-63-4	x	x	x	x
28.024	3-methyl-2-heptanone	2371-19-9		x		
28.024	2-methoxy-4-vinylphenol	7786-61-0				x
28.1672	2,4-decadienal, (E,E)	25152-84-5	x			
29.5943	1-methylpropyl butanoate	97-87-0	x			
29.5943	bis(2,2-dimethyl propyl) disulfide	37552-63-9			x	
29.5943	isobutyl isobutylrate	97-85-8				x
29.8712	o-hexyl-hydroxylamine	4665-68-3			x	
30.3084	1,1'-diol-1,1'-bicyclopentyl	5181-75-9		x		
30.3084	5-hexyldihydro-2(3H)-furanone	706-14-9				x
30.3084	dihydro-5-pentyl-2(3H)-furanone	104-61-0	x			
30.3084	dihydro-5-propyl-2(3H)-furanone	105-21-5			x	
30.6314	butyl butyrate	109-21-7	x		x	
30.6314	isobutyl isobutyrate	74367-31-0		x		x
30.9198	n-decanoic acid	334-48-5	x	x	x	
30.9198	tridecanoic acid	638-53-9				x
30.955	2-(dicyclohexylphosphino)-n,n-diethylethanamine	2359-96-8		x		
32.293	1,3-dimethyl-2-imidazolidinone	80-73-9				x
32.293	dimethyl-carbonocyanidothioic amide	16703-47-2			x	
32.7679	o-methyl s-2-diisoropylaminoethyl ethylphosphonothiolate	162085-94-1			x	
32.7679	3-ethyl,2,2-dimethyloxazolidine	1000142-09-0		x		
32.7679	1-nitrosopiperidine	100-75-4				x
33.4324	2-methyl-8-quinolinol	826-81-3	x	x	x	x
33.9458	2-propylthiazole	17626-75-4	x			
35.0233	3',5'-dimethoxyacetophenone	39151-19-4			x	
35.0233	n-ethyl-2-methyl-5-nitrobenzeneamine	56288-95-0		x		
35.0233	undecanoic acid	112-37-8	x			x
35.5563	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-(E)-3-buten-2-one	79-77-6			x	
35.5563	1-(4-hydroxy-3-methoxyphenyl)- ethanone	498-02-2				x
36.0362	tetrahydro-6-pentyl-2H-pyran-2-one	705-86-2	x	x	x	x
36.5715	3,3-dimethyl-5-phenyl-3h-pyrazole	1000211-16-9	x			
36.5715	N-acetyl-2,4-difluoroaminobenzene	1000130-50-9			x	x
36.5715	n-(3,4-difluorophenyl)acetamide	458-11-7		x		
36.8287	2,4-bis(1,1-dimethylethyl)-phenol	96-76-4	x	x	x	x
36.9856	3,3-dimethylpyrrolidine-2,5-dione	3437-29-4		x		
36.9856	3-amino-2-cyclohexenone	5220-49-5	x			
37.274	dihydroactinidiolide	15356-74-8	x		x	
37.274	ethyl-4-ethoxybenzoate	23676-09-7		x		
38.9953	dodecanoic acid	143-07-7	x	x	x	x
41.1676	benzophenone	119-61-9	x	x	x	x
41.4502	4-methyl-1,6-heptadien-4-ol	25201-40-5				x
41.4502	polyparaben	94-13-3	x			
42.4989	dihydro-5-(2-octenyl)-(Z)-2(3H)-furanone	18679-18-0				x
42.7561	tetrahydro-4,6-dimethyl-2H-pyran-2-one	1000150-22-1		x		
42.7561	tridecanoic acid	638-53-9	x		x	x
43.241	γ dodecalactone	2305-05-7				x
43.241	5-heptyldihydro-2(3H)-furanone	104-67-6			x	
43.241	2,4-dimethylundecane	17312-80-0		x		
44.362	6-heptyltetrahydro-2H-pyran-2-one	713-95-1	x	x	x	x
46.5078	tetradecanoic acid	544-63-6	x	x	x	x
48.6154	1,3,5-triazine-2,4,6(1H,3H,5H)-trione	108-80-5				x

48.6154	1-octadecanethiol	2885-00-9		x		
48.6154	lauric anhydride	645-66-9			x	
49.2142	4-methoxy-1-methyl-bicyclo(2.2.2)octanone	3907-11-7			x	
49.2142	oxacyclotridecan-2-one	947-05-7				x
49.9306	pentadecanoic acid	1002-84-2	x	x	x	x
50.174	tetratriacontane	14167-59-0		x		
51.8237	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1000143-92-4	x		x	x
51.8237	3-methylpentadecane	2882-96-4		x		
52.6647	Z-11-hexadecenoic acid	2416-20-8	x	x	x	x
53.5103	n-hexadecanoic acid	57-10-3	x	x	x	x
54.3467	17-pentatriacontene	6971-40-0	x			
54.3467	octadecanoic acid	57-11-4			x	
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide	1544-50-0	x	x	x	x
55.3296	oxybenzone	131-57-7				x
55.5615	Z-methyl ester-9-hexadecenoic acid	1120-25-8				x
55.5615	E,E-2,13-octadecadien-1-ol	1000131-09-8	x			
55.7576	1-octadecene	506-43-4	x			
55.7576	Z-methyl ester-9-hexadecenoic acid	1120-25-8				x
56.4959	heptadecanoic acid	506-12-7	x	x	x	x
57.6773	17-pentatriacontene	6971-40-0		x		
58.3325	1,2-15,16-diepoxyhexadecane	1000192-65-0				x
58.3325	Z-2-octadecen-1-ol	1000131-11-0	x			
58.9301	(Z,Z)-9,12-octadecadienoic acid	60-33-3	x	x	x	x
59.1043	(E)-9-octadecenoic acid	112-79-8	x	x	x	x
59.8669	octadecanoic acid	57-11-4	x	x	x	x
65.6431	ethyl (all-Z)-5,8,11,14-eicosatetraenoate	1808-26-0	x	x	x	x

Appendix II

Organic Compounds Identified in Female Canid Urine Using SBSE Method

Appendix II – Organic compounds identified in female canid urine using SBSE method

Retention Time [RT]	Compound	Red Wolf Female	Grey Wolf Female
1.2679	acetic acid	x	x
1.5251	1-butanol		x
1.7351	2-pentanone	x	x
1.768	2,3-butanedione	x	
1.9185	propanoic acid		x
1.9993	methyl propyl sulfide	x	x
2.967	2-methyl-1-(methylthio)-propane	x	
3.5105	hexanal		x
4.017	n-butyl methyl sulfide	x	
4.6884	furfural		x
5.2479	diacetamide		x
6.2481	4-heptanone		x
6.313	2-methyl-3-hexanone	x	
6.313	2,4-dimethyl-3-pentanone	x	
6.486	2-(methylthio)-ethanol	x	
7.0972	2-heptanone	x	x
7.432	l-methioninol	x	
8.372	methoxyphenyloxime		x
8.8115	α pinene		x
9.4022	4-methyl-2-heptanone	x	x
9.8428	bicyclo(3.2.0)hepta-2,6-diene		x
10.317	benzaldehyde	X	x
10.42	3-ethylcyclopentanone	x	
10.8454	2-nitro- 1,4-benzenedicarboxamide		x
11.819	2,3-octanedione		x
12.139	phenol	x	
12.2782	hexanoic acid		x
12.704	pentanoic acid	x	
13.327	3-methoxy-2-butonal	x	
13.373	4-cyanocyclohexene		x
13.546	2-ethenyl-6-methylpyrazine	x	
13.892	hexane	x	
14.5312	salicylaldehyde	x	x
14.642	1,2-diethiepane	x	
15.1323	2-methyl-2-hexanol		x
15.7852	acetophenone	x	x
16.3448	1-nonene		x
16.5213	p-tolualdehyde		x
16.626	3-methylbenzaldehyde	x	
17.076	3-methyl-2-heptanone	x	
17.1788	5-hexenoic acid		x
17.3115	1-(2,4-dimethyl-furan-3-yl)-ethanone		x
17.9056	nonanal	x	x
18.3486	furan		x
19.0639	benzyl methyl ketone		x
19.8137	4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2ol		x
21.4588	1,2-benzenedicarbonitrile		x
22.152	propylpropanedioic acid	x	
22.9713	(1S) 4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2-one		x
24.2749	quinoline	x	x
25.132	1,3-bis(1,1-dimethylethyl)-benzene	x	x
26.6629	nonanoic acid	x	x
26.674	(1S-endo)(4,7,7-trimethyl-3 bicyclo [2.2.1] heptanyl) acetate		x
27.1463	3-methyl-1-penten-4-yn-3-ol		x
27.5535	2-methyl-quinoline	x	x
28.1672	2,4-decadienal, (E,E)		x
29.5943	1-methylpropyl butanoate		x
30.6314	2-methyl-3-hydroxy-2,4,4-trimethylpentyl propanoic acid		x
30.9198	n-decanoic acid		x

33.4324	2-methyl-8-quinolinol	x	x
33.9458	2-isobutylthiazole		x
35.0233	butylated hydroxyanisole		x
35.257	cyclododecane	x	
35.258	nonyl-cyclopropane	x	
35.5563	(E) 6-(2-butenyl)-1,5,5-trimethylcyclohexene		x
36.0362	tetrahydro-6-pentyl-2H-pyran-2-one		x
36.5715	N-acetyl-2,4-difluoroaminobenzene		x
36.8287	2,4-bis(1,1-dimethylethyl)-phenol	x	x
37.274	dihydroactinidiolide	x	x
38.442	7-hydroxy-4-methylchromen-2-one	x	
38.9953	dodecanoic acid		x
39.353	(1R-4R-6R-10S)-9-methylene-4,12,12,-trimethyl-5-oxatricyclo(8.2.0.0)4,6 dodecane		x
41.095	diphenylamine	x	
41.1676	benzophenone		x
41.4502	polyparaben		x
42.525	tributyl phosphate	x	
42.7561	tridecanoic acid		x
43.241	γ dodecalactone		x
43.921	cyclododecane	x	
44.362	6-heptyltetrahydro-2H-pyran-2-one		x
44.994	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	x	
45.698	3,6-dihydro-3-hydroxy-6-(1-methylethoxy)-2H-pyran-2-methanol	x	
46.5078	tetradecanoic acid	x	x
49.9306	pentadecanoic acid		x
51.8237	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione		x
51.87	9,10-dihydro-9,9-dimethylacridine	x	
52.6647	Z-11-hexadecenoic acid	x	x
53.5103	n-hexadecanoic acid	x	x
53.762	6,7-dimethoxy-2H-1-benzopyran-2-one	x	
54.3467	octadecane		x
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide	x	x
55.5615	Z-11-tridecen-1-ol acetate		x
55.7576	Z-8-methyl-9-tetradecenoic acid		x
56.4959	3a,6,6,9a-tetramethyl-1,4,5,5a,7,8,9,9b-octahydro-benzo [E] benzofuran-2-one		x
58.9301	(Z,Z)-9,12-octadecadienoic acid	x	x
59.057	oleic acid	x	
59.1043	(E)-9-octadecenoic acid	x	x
59.8669	octadecanoic acid	x	x
63.268	hexatriacontane	x	
65.425	9-octylheptadecane	x	
65.6431	ethyl (all-Z)-5,8,11,14-eicosatetraenoate		x
65.668	undecylbenzoate	x	

Appendix III

Organic Compounds Identified in Red Wolf Urine Using SBSE Method

Appendix III – Organic compounds identified in both red wolf male and female urine using SBSE method

Retention Time [RT]	Compound	Red Wolf Male	Red Wolf Female
1.4478	acetic acid	x	x
1.7351	2-pentanone	x	x
1.768	2,3-butanedione		x
1.9185	propanoic acid	x	
1.9993	methyl propyl sulfide	x	x
2.967	2-methyl-1-(methylthio)-propane		x
3.5105	2-oxo-3-methyl-cis-perhydro-1,3-benzoxazine	x	
3.9674	n-butyl methyl sulfide	x	x
4.6884	furfural	x	
5.2479	3-methyl-2-hexanone	x	
6.2481	4-heptanone	x	
6.313	2-methyl-3-hexanone		x
6.313	2,4-dimethyl-3-pentanone		x
6.486	2-(methylthio)-ethanol		x
7.0972	2-heptanone	x	x
7.432	l-methioninol		x
8.372	methoxyphenyloxime	x	
9.4022	4-methyl-2-heptanone	x	x
9.8428	2,4-diphenyl-2,3-dihydro-1,5-benzothiazepine	x	
10.317	benzaldehyde	x	
10.42	3-ethylcyclopentanone		x
10.8454	2-nitro- 1,4-benzenedicarboxamide	x	
11.819	3-octanone	x	
12.139	phenol		x
12.2782	pentanoic acid	x	x
13.327	3-methoxy-2-butonal		x
13.546	2-ethenyl-6-methylpyrazine		x
13.892	hexane		x
14.1321	2,2,6-trimethylcyclohexanone	x	
14.5312	salicylaldehyde	x	x
14.642	1,2-diethiepane		x
15.7852	acetophenone	x	x
16.5213	p-tolualdehyde	x	
16.626	3-methylbenzaldehyde		x
16.949	3,5-dimethyl-2-octanone	x	
17.1788	2-nonanone	x	
17.9056	nonanal	x	x
21.4588	benzoic acid	x	
22.091	hexanoic acid	x	
22.152	propylpropanedioic acid		x
22.9713	decanal	x	
23.0635	3,4-dimethyl-2-hexanone	x	
23.5584	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	x	
23.756	dihydro-3-pentyl-2(3H)-furanone	x	
24.2749	quinoline	x	x
25.132	1,3-bis(1,1-dimethylethyl)-benzene	x	x
26.6629	nonanoic acid	x	x
27.5535	2-methyl-quinoline	x	x
28.024	3-methyl-2-heptanone	x	x
30.3084	1,1'-diol-1,1'-bicyclopentyl	x	
30.6314	isobutyl isobutyrate	x	
30.9198	n-decanoic acid	x	
30.955	2-(dicyclohexylphosphino)-n,n-diethylethanamine	x	
32.7679	3-ethyl,2,2-dimethyloxazolidine	x	
33.4324	2-methyl-8-quinolinol	x	x
35.0233	n-ethyl-2-methyl-5-nitrobenzeneamine	x	
35.258	nonyl-cyclopropane		x
36.0362	tetrahydro-6-pentyl-2H-pyran-2-one	x	

36.5715	n-(3,4-difluorophenyl)acetamide	x	
36.8287	2,4-bis(1,1-dimethylethyl)-phenol	x	x
36.9856	3,3-dimethylpyrrolidine-2,5-dione	x	
37.274	ethyl-4-ethoxybenzoate	x	
37.427	(R) 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone		x
38.442	7-hydroxy-4-methylchromen-2-one		x
38.9953	dodecanoic acid	x	
41.095	diphenylamine		x
41.1676	benzophenone	x	
42.525	tributyl phosphate		x
42.7561	tetrahydro-4,6-dimethyl-2H-pyran-2-one	x	
43.241	2,4-dimethylundecane	x	
43.921	cyclododecane		x
44.362	6-heptyltetrahydro-2H-pyran-2-one	x	
44.994	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol		x
45.698	3,6-dihydro-3-hydroxy-6-(1-methylethoxy)-2H-pyran-2-methanol		x
46.5078	tetradecanoic acid	x	x
48.6154	1-octadecanethiol	x	
49.9306	pentadecanoic acid	x	
50.174	tetratriacontane	x	
51.8237	3-methylpentadecane	x	
51.87	9,10-dihydro-9,9-dimethylacridine		x
52.6647	Z-11-hexadecenoic acid	x	x
53.5103	n-hexadecanoic acid	x	x
53.762	6,7-dimethoxy-2H-1-benzopyran-2-one		x
54.739	2,6-dichloro-N-(2-hydroxy-1,3 dioxo-2,3-dihydro-1H-inden-2-yl) benzamide	x	x
56.4959	heptadecanoic acid	x	
57.6773	17-pentatriacontene	x	
58.9301	(Z,Z)-9,12-octadecadienoic acid	x	x
59.057	oleic acid		x
59.1043	(E)-9-octadecenoic acid	x	x
59.8669	octadecanoic acid	x	x
63.268	hexatriacontane		x
65.425	9-octylheptadecane		x
65.6431	ethyl (all-Z)-5,8,11,14-eicosatetraenoate	x	
65.668	undecylbenzoate		x