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SCENT MARKING IN A HIGHLY SOCIAL MAMMALIAN SPECIES, THE SPOTTED HYENA, *CROCUTA CROCUTA*

presented by

KEVIN ROBERT THEIS

has been accepted towards fulfillment
of the requirements for the

| Doctoral | degree in | Zoology and Ecology, Evolutionary Biology and Behavior |
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Major Professor's Signature

12/8/08

Date

# SCENT MARKING IN A HIGHLY SOCIAL MAMMALIAN SPECIES, THE SPOTTED HYENA, *CROCUTA CROCUTA*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Zoology and Ecology, Evolutionary Biology and Behavior

2008

ABSTRACT

SCENT MARKING IN A HIGHLY SOCIAL MAMMALIAN SPECIES, THE SPOTTED HYENA, *CROCUTA CROCUTA*

By

Kevin Robert Theis

Previous research on the functions of mammalian scent marking has often focused on marking by adult males of solitary species, in territorial contexts. However, it has become clear that the functions of scent marking vary not only among species, but also among different age, sex, reproductive and social classes within a single species. Here I evaluated multiple functional hypotheses for scent marking ('pasting') in the highly social spotted hyena, *Crocuta crocuta*. I used naturalistic observational data to ascertain the social contexts in which pasting occurred, and conducted scent discrimination experiments with free-living hyenas to determine the information content of paste.

In demonstrating that hyenas discriminate between the scents of clanmates and others, I reaffirmed a territorial function for pasting. Pasting also appeared to function in advertising dominance status for adult males, but not females. Rates of pasting among males were dependent upon social rank, and males pasted most often when their social partners were primarily other males. For adult females, pasting appeared to function in maintaining social cohesion within clans. Female pasting rates were highest at dens; the den is the social center of each hyena clan. Additionally, adult females investigated female more than male scents, and females that engaged in extended absences from their clans pasted more often upon returning to their clans than they did prior to their departing. Adult females did not paste to advertise their sexual receptivity to males, as their pasting rates were highest during pregnancy, not late lactation, when females are

most likely to be sexually receptive. Although information about female reproductive state is clearly available in paste, its functional significance, if any, remains unknown. Among adult males, pasting did not appear to advertise availability for reproductive opportunities, as male pasting rates did not predict their relative reproductive success. Lastly, an ontogenetic investigation of scent marking revealed that hyena cubs 'pasted' as often as older conspecifics, yet they likely did not deposit scents because hyenas did not consistently produce paste until their third year of life. Instead, by 'pasting,' cubs may be acquiring group-specific odors and bacteria that facilitate their being recognized as group members before they are widely known as individuals to conspecifics.

Symbiotic bacteria in the scent glands of mammals are believed to be responsible for generating many of the semiochemicals their hosts use in communication. Members of mammalian social groups might, via cross-infection, come to share similar bacterial communities in their scent glands. Consequently, they might emit group-specific odors that, at the functional level, promote social cohesion. Here I tested an hypothesis suggesting a bacterial mechanism for group-specific odors using free-living spotted hyenas and culture-independent sampling methods (16S rRNA gene surveys). The scent glands of hyenas contain many previously uncultured anaerobes, mostly species of *Peptostreptococcus*, *Anaerococcus*, *Propionibacterium* and *Corynebacterium*. Members of these genera produce compounds believed to figure prominently in hyena scent marking. Also, graphical and statistical analyses illustrated that, although not universal, group-specific differences in the structure of symbiotic bacterial communities did exist within the scent glands of hyenas, thus supporting the hypothesis that bacteria are responsible for the production of group-specific odors in this species.

ACKNOWLEDGMENTS

As a young adult, I followed a meandering academic and professional path. Along the way I benefited from the generous guidance of several mentors, but one deserves special mention. The Reverend Harold Ridley was a towering, sophisticated, masterfully intellectual man, who first showed me that genuine concern and generosity distinguish great teachers from ordinary ones. I cherished Father Ridley's aura, his warmth, his dedication to his students and, selfishly, his belief in the capacity of my mind. I wish dearly I could share this accomplishment with him. As I cannot, I dedicate it to him.

The meandering path I followed eventually led me to behavioral biology, and to Bill Shields. I have lauded Bill previously when endorsing him for a Distinguished Teaching Professorship, so will refrain from doing so now. Suffice it to say that Bill is the most innovative and effective teacher I have ever observed. I am deeply indebted to him for providing me an ideal toward which to strive. Additionally, were it not for Bill, I'd never have met Kay Holekamp.

There is no evident reason why Kay accepted me into her prestigious lab. Regardless, I am eternally grateful she did. Through Kay's graciousness I have lived beside and studied large carnivores in the African bush, and through her guidance I have begun developing into an independent behavioral biologist. Kay is tireless in her attempts at furthering the careers of her graduate students. She secured incredibly valuable teaching opportunities for me when I wasn't yet deserving, and she has repeatedly provided me with financial support when under no obligation to do so. Kay routinely answers emails in minutes, not days, and returns meticulously edited manuscript drafts in days, not weeks. If I eventually possess a lab of my own, the respect and treatment I

afford my students will be due in large part to the example Kay has set for me, and any success I achieve will be a direct result of having had Kay in my corner.

My intention when starting at MSU was to study vocal communication in hyenas. However, after I attended a few meetings of the ODOR group, I shifted my focus to chemical communication. The ODOR group contributed greatly to the development of this dissertation. John Stoltzfus was the impetus behind the microbial work, and two ODOR members were so broadly influential in the development of my research that I requested they serve on my advisory committee. Heather Eisthen has been strongly supportive throughout my graduate career. She has repeatedly made time for discussion with me and always provided detailed edits of my writing. She has also often been a welcome source of perspective. Jim Miller was instrumental in selling me on the importance of chemical stimuli in behavioral biology, in my developing an understanding of the scientific method, and in my recognizing the importance of focusing on hypothesis-driven problems. Tom Getty is the other member of my advisory committee and, although not an ODOR member, he was instrumental during the formative stages of my dissertation as well. Tom is very adept at broad-level, conceptual thinking, and his contributions to my understanding of the fields of animal communication and behavioral ecology are greatly appreciated.

Barb Lundrigan and Tom Schmidt also substantially enhanced my professional development. Barb offered insightful discussion on mammalian scent marking and inspired me to continually improve as a teacher. Tom is an incredibly approachable and, thankfully, patient man. Similar to Kay, Tom took me into his lab when reason must surely have been screaming at him not to. Tom has already invested much time, thought,

and many resources in me and my research. The microbial work in this dissertation simply would not have been possible without him. I am deeply indebted to Tom for his continued generosity and intellectual curiosity. Each of his students, but especially Brad Stevenson, Stephanie Eichorst, and Zarraz Lee, graciously provided technical assistance.

My dissertation work is a small part of a long term research project addressing the behavioral ecology of spotted hyenas in the Masai Mara National Reserve (MMNR), Kenya. The Mara Hyena Project has had numerous contributors, many of whom collected data analyzed in this dissertation. Laura Smale, co-founder of the project, deserves specific mention, as does Russ Van Horn, who provided the paternity analyses. My enjoyment of the Mara was heightened by my relationships with John Keshe, James Kerembe, Moses Sairowa, Stephen and Lesingo Nairori. I am indebted to all members of the Holekamp lab. Kay is such a prolific mentor that I cannot name them all. However, three must be acknowledged directly. Joe Kolowski offered humor, an ear upon which to vent frustration and, occasionally, an excuse for a beer and an NFL game. Terri McElhinny provided frequent moral support. Her boundless optimism proved a comforting compliment to my chronic cynicism. Most deserving of my gratitude, however, is Jaime Tanner. She was critical to my accomplishing my research objectives in the Mara and at home, and she has become a dear friend. She has frequently been quick to forgive my shortcomings as both a colleague and a friend. I simply cannot thank her enough.

I have benefited from the assistance of Pat Bills, our resident computer guru and a welcome source of conversation. Also, I have had numerous undergraduate assistants: Anna Heckla, Joey Verge, Jessi Smashey, Elizabeth Brown, Kelly Pyle, Amy Kuczynski

and Laura Cisneros. It was a true delight to watch Anna, Joey and Jessi progress from collecting data to asking biological questions and formulating hypotheses of their own. Of course, science requires money, and I have benefited from the generosity of the following funding sources: National Science Foundation (awards to K.E. Holekamp), Office of the Graduate School, College of Natural Science, Department of Zoology, Program in EEBB, Shaver Fellowship Committee, American Society for Microbiology, and Sigma Xi. I must also thank the Office of the President of Kenya and the Senior Warden of the MMNR for permission to conduct this research.

Lastly, I thank my family, which has always offered me unconditional support. They remained patient as I meandered my way through life, seemingly delaying adulthood. I cannot remember a single instance of them telling me, or even hinting at the possibility, that I could not accomplish something. I have been blessed with an unbelievably supportive family by marriage as well. My mother-in-law even sheltered and fed me while I returned to school for a second bachelor's degree. Of course, this dissertation would not have been possible without the full support of my wife, Lisa. She has had to assume many of my parental responsibilities while I have been in graduate school. I am immensely grateful to her, and to our beautiful daughters Juliana Rose and Karissa Ann, for their understanding and patience.

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# GENERAL INTRODUCTION

The objectives of this introductory essay are to provide a working definition of animal communication, to argue the continued importance of animal communication studies, to emphasize the particular value of chemical communication studies, and to explain why free-living spotted hyenas are intriguing subjects with which to expand our understanding of the mechanistic, developmental, and functional explanations of mammalian scent marking. This essay concludes with a discussion of my research objectives, including synopses of the data chapters constituting my thesis.

## What is animal communication?

Since a widely accepted definition of animal communication has not yet been developed (Maynard Smith & Harper 2003), it is important for individual researchers to provide clear, working definitions of communication early in their writings. Animal communication is here defined as the transmission of information from a sender to a receiver that effects a change in the receiver's behavior and/or physiology, thereby benefiting, on average, both the sender and receiver (Dusenbery 1992; Bradbury & Vehrencamp 1998). Information is transmitted via 'signals' and effected changes in receivers are labeled 'responses.' Given that signalers and receivers seldom have identical interests, each party often has a substantially different view of what constitutes an ideal response (Krebs & Dawkins 1984). Consequently, signalers are often perceived as attempting to 'manipulate' receivers, while receivers are seen as discriminating 'mind-readers,' selectively responding only to honest signals (Krebs & Dawkins 1984). If a signal consistently conveys dishonest information, then receivers who indiscriminately

respond to the signal do so with consequent detriment to their fitness. Therefore, there is strong selective pressure on receivers to ensure that they respond only to signals that transmit honest, reliable information (Krebs & Dawkins 1984; Bradbury & Vehrencamp 2000). Consequently, signaling systems based on unreliable signals are not evolutionarily stable (Johnstone 1997; Bradbury & Vehrencamp 1998, Maynard Smith & Harper 2003), and signals typically provide honest information about senders and/or their social and physical environments (e.g. Gosling et al. 2000; Blas et al. 2006; Forsman & Hagman 2006; Lopez et al. 2006; Velando et al. 2006; Vanpe et al. 2007; Villasenor & Drummond 2007; but see Elwood et al. 2006; Setchell et al. 2006).

**Why is the study of animal communication important?**

Although the study of animal communication was heralded in Darwin's *The Expression of the Emotions in Man and the Animals* (1872), it was first formally pursued in the early twentieth century by the classical ethologists (reviewed by Burkhardt 2005), and continued by their students (e.g. Marler 1967; Kruuk 1972). It remains a vibrant and robust field of study for four primary reasons (Bradbury & Vehrencamp 1998). First, communication plays a central role in animal societies. Many animals participate in societies because group-living affords improved defense of resources, reduced predation risk, increased foraging efficiency, and enhanced reproductive output (Alcock 2005). However, social animals must also deal with the substantial costs associated with a gregarious lifestyle. These costs include increased competition for resources, greater investment and risk of injury in establishing and maintaining dominance hierarchies, higher prevalence of disease and parasite transmission, and an increased likelihood of

mating with close kin (Alcock 2005). Communication has been lauded as the "glue that holds animal societies together" (Bradbury & Vehrencamp 1998, p. 5), because it facilitates the benefits, and modulates the costs, of living in groups. Although communication is often most conspicuous among highly social animals, even solitary ones require it to maintain spacing between competitors and to bring potential mates together during reproductive periods (Bradbury & Vehrencamp 1998). In fact, communication is ubiquitous throughout the animal kingdom, leading one preeminent scholar to proclaim that "nothing would work in the absence of communication" (Hauser 1996, p. 1).

The second reason researchers study animal communication is to ascertain the full extent of information that animals convey to each other, and to elucidate the mechanisms underlying the production, reception and interpretation of signals (Dusenbery 1992; Bradbury & Vehrencamp 1998). Numerous studies have demonstrated that animals communicate information about their species, group, sex and individual identity, their reproductive, social, and motivational status, and their immediate social and physical environment (Bradbury & Vehrencamp 1998; Maynard Smith & Harper 2003). Ascertaining the information that animals communicate to others allows behavioral ecologists to formulate hypotheses about the adaptive significance, and optimization, of signaling for different animal species, age groups and sex classes. Furthermore, in addition to increasing our understanding of animal physiology and sensory ecology, attempts at elucidating the mechanisms underlying animal signaling encourage collaborations among researchers with disparate scientific backgrounds, most notably engineering, physics, chemistry, microbiology, neuroscience, behavioral ecology and

psychology (see proceedings from the Sackler Colloquium of the National Academy of Sciences, Irvine, CA, 2003; Chemical Signals in Vertebrates XI, Chester, England, 2006). Collaborations among researchers with different backgrounds likely promote theoretical, methodological, statistical, and technological innovation.

A third reason the study of animal communication remains productive is that it can serve as a tool for exploring general evolutionary principles (Bradbury & Vehrencamp 1998). One of the primary interests of the classical ethologists was the evolution of signals (Kruuk 2003; Burkhardt 2005). Over the last half-century their pioneering efforts have mushroomed into a myriad of studies on evolutionary topics in communication, such as signal optimization in response to background noise and predator eavesdropping (e.g. Ryan et al. 1982; Bayly & Evans 2003; Aubin 2004; Brumm et al. 2004; Remage-Healey et al. 2006; Ord et al. 2007; Poesel et al. 2007), and the role of sexually selected signals in reproductive isolation and speciation (e.g. Sorenson et al. 2003; Kingston & Rossiter 2004; Boul et al. 2007; Bradbury & Vehrencamp 1998).

The fourth reason animal communication studies are important is that they have practical applications (Bradbury & Vehrencamp 1998). Studies of insect pheromones have lead to the development of pheromone bait-traps that help control agricultural and forest pests (e.g. Subchev et al. 2004; Jeans-Williams & Borden 2006; Ibeas et al. 2007). Recently, behavioral ecologists have begun investigating whether similar tactics can be employed to minimize carnivore-livestock conflicts around nature reserves (e.g. Parker 2006). Conservationists have also utilized audio playback experiments and animal signaling activity to estimate the population size of endangered species and to determine species diversity in vulnerable areas (e.g. Apollonio et al. 2004; Barbraud & DeLord

2006; Geissmann & Nijman 2006; Ryan et al. 2006; Barea-Azcon et al. 2007). Lastly, knowledge gained about how animals communicate with conspecifics has lead to the improved welfare of captive animals (e.g. Olsson et al. 2003; Schulte et al. 2007), and enhanced the success of captive breeding programs (e.g. Fisher et al. 2003; Roberts & Gosling 2004; Zhang et al. 2004).

**Why study chemical communication, in particular?**

If the value of animal communication studies is recognized, then the value of chemical communication studies, in particular, should be acknowledged as well, given that chemical signaling is the most ancient and widely utilized form of communication in the animal kingdom (Agosta 1992; Dusenbery 1992; Doty 1995; Wyatt 2003). One challenge that has historically hindered the investigation of chemical communication is that human sensory systems, although well-adapted for perceiving many of the vocal and visual signals generated by animals, are relatively insensitive to the chemical messages that figure so prominently in the umwelten of most animal species, including the majority of non-human mammals (Blaustein 1981; Penn & Potts 1998). For example, consider the despondent reaction of George Schaller when he realized, after months of studying wild giant pandas, that he and they occupied disparate sensory worlds. "How would I ever understand pandas? They moved from odor to odor, the air filled with important messages where I detected nothing" (1993, p. 99). While Schaller's observation poignantly illustrates the challenge associated with studies of chemical communication, it also serves to intrigue many scientists.

If the presumption is correct that we fail to perceive the vast majority of communications that occur around us, then the prospect of utilizing technological innovations and the systematic observation of animals to discover how they communicate via scent is, for myself and others, a highly attractive endeavor. Over the past few decades, chemical and behavioral ecologists have sought to document the full extent of information communicated via chemicals among animals, to determine how these odors are produced, sensed and interpreted, and to demonstrate how chemical signaling is evolutionarily adaptive for a wide range of species (Bradbury & Vehrencamp 1998; Wyatt 2003). One form of chemical signaling that has received a great deal of investigation is mammalian scent marking (see Johnston 2003; Hurst & Beynon 2004).

Scent marking is the deliberate deposition of urine, feces and/or the products of secretory glands in the environment, and is one of the primary forms of signaling employed by mammals (Johnson 1973). A unique characteristic of scent marks is that they persist as viable messages for considerable periods of time where they were deposited, even after their sender has departed (Wynne-Edwards 1962; Marler 1967; Eisenberg & Kleiman 1972). Consequently, although communication is traditionally described as occurring within a dyad (Bradbury & Vehrencamp 1998), scent marking is most appropriately viewed as an act of communication conducted within a network of individuals (Hurst 2005). Communication networks exist whenever signals travel farther than the average spacing among individuals in the signaling system (McGregor 1993; McGregor 2005). Therefore, the likelihood of network formation is dependent upon both population density and signal properties, specifically broadcast distance and fade-out time. Due to limited broadcast distance, visual, tactile and electrical signaling are

generally restricted to individuals in immediate proximity of one another (Dusenbery 1992; Bradbury & Vehrencamp 1998). A dyadic representation of communication in these modalities is often, therefore, sufficient. Audible signals, however, can travel distances far in excess of the average spacing between individuals and thus potentially provide information to multiple receivers over a large area (e.g. Grafe 2005; McComb & Reby 2005). Some chemical signals also travel considerable distances on air or water currents and may be perceived by numerous receivers spaced far apart (Wilson & Bossert 1963; Elkinton & Carde 1984). Scent marks, however, do not travel far, yet their persistence makes them available to multiple receivers over time, and, therefore, they are best considered as occurring within a network (Hurst 2005). The distinction here is not trivial, as scent marks cannot be directed toward specific recipients. Rather, once deposited, they are available to all individuals who happen upon them (Hurst 2005). Therefore, there has likely been strong selection pressure to ensure that the information contained in scent marks is appropriate for communication with all animals in the area rather than one specific individual (Hurst 2005).

Mammalian scent marks can convey information about the donor's species, group, sex, age, genetic and individual identity, as well as its reproductive, social, nutritional and health status (reviewed by Penn & Potts 1998; Gosling & Roberts 2001; Wyatt 2003; Hurst & Beynon 2004; Hurst 2005). Communicating this information to conspecifics functions to establish and maintain territories, advertise dominance and reproductive status to competitors and potential mates, facilitate social cohesion, and increase foraging efficiency (reviewed by Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973; Thiessen & Rice 1976; Gorman & Trowbridge 1989; Wyatt 2003; Hurst

2005; Lewis 2006). Scent marking is therefore an intriguing form of communication to study, as scent marks potentially contain a wealth of information about their donors and the use of scent marks likely serves different adaptive functions for each age, sex and social class that deposits and/or accesses the information available in them (Yahr 1983; Hurst 2005).

However, historically, the adaptive function of mammalian scent marking that has received the vast majority of attention is territorial maintenance (see Gosling 1982; Gorman & Trowbridge 1989; Gosling & Roberts 2001). This over-emphasis on territorial scent marking has occurred despite recommendations that research emphases be more comprehensive (e.g. Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973; Bearder & Randall 1978). The argument of these authors was not that scent marking is afforded too prominent a role in territorial maintenance, but rather that emphasizing a single function to the exclusion of others potentially underestimates the complexity of mammalian chemical signaling systems and the number of functions they serve. Recently, mammalian scent marking studies have become more comprehensive, and as a result, we now have evidence that the functions of scent marking vary not only among mammalian species, but also among different age, sex, reproductive and social classes within the same species (e.g. Drea et al. 2002; White et al. 2003; Palagi et al. 2004; Hurst 2005; Lewis 2006; Mateo 2006; Scordato & Drea 2007). Furthermore, it has become apparent that we have a particularly poor understanding of chemical communication in highly social species (Scordato & Drea 2007). The broad objective of my dissertation research is therefore to provide a comprehensive understanding of the scent marking behavior of the

gregarious spotted hyena, *Crocuta crocuta*, a large, keystone predator found throughout much of sub-Saharan Africa (Estes 1992).

**Why study scent marking in the spotted hyena?**

Spotted hyenas live in complex social groups, called clans, that typically contain 40 – 80 individuals (Kruuk 1972; Trinkel et al. 2007). Members of a clan cooperatively defend territories from neighboring hyenas, and they defend ungulate kills from both other hyenas and African lions, *Panthera leo* (Kruuk 1972; Mills 1990; Henschel & Skinner 1991; Boydston et al. 2001). Similar to the complex societies of many cercopithecine primates, hyena clans contain multiple breeding males and multiple overlapping generations of females (Holekamp et al. 2007). Another characteristic hyena clans share with some primate societies is that they are structured by linear dominance hierarchies (Frank 1986b; Holekamp et al. 2007). In contrast to most mammalian social systems, a hyena's position within its clan's hierarchy is not determined by its fighting ability. Instead, natal hyenas inherit their mother's rank (Frank 1986b; Holekamp & Smale 1991, 1993; Smale et al. 1993; Engh et al. 2000). Additionally, adult immigrant males, all of whom are lower-ranking socially than natal animals, queue for social status with other immigrants (Smale et al. 1997; East & Hofer 2001). Interestingly, the dominance queue among immigrant males is maintained despite males exhibiting very low rates of aggression towards one another (Frank 1986b; Smale et al. 1997; East & Hofer 2001). The low rates of aggression among males are interesting because, as with female hyenas (Holekamp et al. 1996), a male's position within the clan's hierarchy has a substantial effect on his reproductive success (Engh et al. 2002; Hofer & East 2003).

Therefore, a substitute for aggression in maintaining the stability of male dominance queues likely exists. Communication is a likely candidate.

Given the complexity of spotted hyena societies, it is not surprising that hyenas emit a rich array of vocal, visual and chemical signals (Blumstein & Armitage 1997; Freeberg 2006; Kruuk 1972; Mills 1990). *Crocuta*'s vocal repertoire includes long-distance whooping, aggression-elicited giggling, groaning over cubs, and alarm rumbling (Kruuk 1972; East & Hofer 1991a). Spotted hyenas also exhibit numerous facial, postural and genital displays that can indicate agonistic motivation and intent, sexual interest, and/or general arousal (Kruuk 1972; East et al. 1993). The vocal and visual signals of spotted hyenas are routinely utilized to communicate information among members of the same clan (Kruuk 1972; Mills 1990; East & Hofer 1991b; East et al. 1993; Holekamp et al. 1999; Theis et al. 2007).

The potential chemical signaling behaviors of spotted hyenas include rolling, scent-rubbing, forepaw scraping, defecating at latrines and anal gland 'pasting' (Kruuk 1972; Bearder & Randall 1978; Mills 1990; Henschel & Skinner 1991). Pasting is the most common and conspicuous of these behaviors. It is a form of scent marking exhibited by all four extent members of Family Hyaenidae, during which hyenas drag their extruded anal pouches over grass stalks or other objects in the environment (Kruuk 1972; Mills 1990; Sliwa & Richardson 1998). As hyena anal pouches contain a pasty mass, consisting of degenerated epidermal cells bound by a waxy substratum, pasting results in hyenas depositing a thin layer of anal gland secretion near the tops of grass stalks (Matthews 1939; Kruuk 1972; Mills 1990). Among spotted hyenas, pasting is sometimes repeated multiple times on the same stalk (Kruuk 1972). Occasionally other hyenas join

in as well, adding their secretion to the same substrate, a phenomenon called 'overmarking' (Kruuk 1972; Mills 1990).

Interestingly, although there is a robust, positive correlation between dominance status and scent marking behavior in most mammals (Ralls 1971), in *Crocuta* societies it is the immigrant males, the lowest-ranking members of the clan, who scent mark most frequently (Mills & Gorman 1987). Given this anomaly, and the fact that vocal and visual communication figure prominently in modulating hyena social interactions within clans, it has been suggested that spotted hyena pasting behavior is utilized exclusively for inter-group communication, i.e. maintaining territories (Gorman & Mills 1984). However, this restricted view of pasting is unwarranted. As will be shown in this dissertation, pasting is performed by all spotted hyenas, regardless of age or sex. Additionally, it is exhibited in a variety of social and behavioral contexts (Kruuk 1972). Therefore, while pasting certainly has a territorial function (Kruuk 1972; Mills & Gorman 1987; Henschel & Skinner 1991; Boydston et al. 2001), as does most mammalian scent marking, pasting likely also has additional intra-clan functions that have yet to be discovered (Hofer et al. 2001; Drea et al. 2002). The first aim of my dissertation research is to ascertain the functions of pasting for all age, sex, social and reproductive classes of spotted hyenas by systematically observing the frequency and context of pasting behavior among free-living subjects, and by conducting scent discrimination experiments with these wild hyenas as well, to determine the potential information communicated via paste. The second aim of my dissertation research is to examine the role of bacteria in generating the information contained in spotted hyena anal gland secretions. The justifications and approaches for achieving these aims are discussed in the chapter synopses that follow.

Throughout the remainder of this dissertation I have employed the pronoun "we" in favor of "I," because, although I assume ownership of the original ideas and analyses included in this document, data collection has truly been a collective effort. Many of the data presented here were extracted from a long-term (currently 19 years), longitudinal study of spotted hyena behavioral ecology that has been conducted by Dr. Kay Holekamp and her many colleagues, students, and research assistants. Other data were generated through collaboration with experts in other fields of study, without whom I would have been unable to achieve my research objectives. Furthermore, each of my dissertation chapters has been, or will be, submitted as a multiple-author manuscript.

**Synopses of dissertation chapters**

**Chapter One**

Among the hundreds of investigations of mammalian scent marking, only a few have substantively addressed the development of the behavior in juveniles (e.g. French & Cleveland 1984; Woodmansee et al. 1991; Sliwa 1996). It is possible that the lack of information on the ontogeny of scent marking is due to the behavior not being observed to an appreciable degree until sexual maturity in many mammalian species (Yahr 1983). However, we cannot be sure, as very few studies have indicated that juvenile scent marking was considered, and not observed (e.g. Lewis 2006). With greater emphasis recently being placed on understanding the operation of natural selection during juvenile life stages in mammals (Holekamp & Smale 1998; Altmann & Altmann 2003), investigating the ontogeny of scent marking in mammalian species in which the behavior

is commonly observed among juveniles will provide us with a more comprehensive understanding of chemical communication in general.

All spotted hyenas frequently paste, regardless of age, although juveniles may do so ineffectively as they reportedly do not produce secretions in their anal glands until they are 12-18 months old (Kruuk 1972). If Kruuk's observation is accurate, then pasting by juvenile hyenas presents a Darwinian puzzle (Alcock 2005), in that performing ineffectual behaviors typically reduces one's fitness. Although unable to further our understanding of pasting by cubs (<1 yr.), a preliminary investigation by Woodmansee et al. (1991) of the ontogeny of pasting in captive hyenas revealed an increase in scent marking as hyenas reached puberty (~24 mos.), and a modest correlation between social status and marking rate at this time as well. These results suggest that the scent marking behavior of older juvenile spotted hyenas adheres to mammalian norms, and potentially functions in communicating dominance status and reproductive condition to competitors and potential mates (Ralls, 1971; Johnson 1973; Yahr 1983). In this chapter, we study the development of pasting behavior in free-living spotted hyenas in the Masai Mara National Reserve, Kenya (MMNR). This study builds upon the preliminary ontogenetic investigation of pasting in captive hyenas (Woodmansee et al. 1991), by including larger sample sizes in natural (as opposed to highly artificial) rearing conditions, and by more strongly emphasizing the scent marking behavior of cubs.

The specific objectives of this chapter are to: 1) accurately determine the age at which spotted hyenas begin consistently producing secretions in their anal glands, 2) describe how rates of juvenile pasting and overmarking vary during development, 3) evaluate the effect of social rank on pasting behavior among juveniles, 4) identify the

behavioral contexts in which pasting occurs among juvenile spotted hyenas, and 5) consider whether juvenile pasting is likely an adaptive behavior, and if so, what functions it potentially serves.

These objectives will be achieved by two means. First, we will examine the anal pouches of spotted hyenas anaesthetized in the MMNR, for the presence of paste secretions. These hyenas will be either known-age animals or individuals in which we can accurately estimate ages via patterns of tooth wear. Second, we will systematically observe the pasting behavior of numerous individually-known hyenas from birth through reproductive maturity to obtain information on scent marking rates and the behavioral and social contexts that elicit pasting.

**Chapter Two**

Mammalian scent marking can function to establish and maintain territories, facilitate social cohesion, increase foraging efficiency, and advertise dominance and reproductive status to competitors and potential mates (reviewed by Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973; Thiessen & Rice 1976; Gorman & Trowbridge 1989; Wyatt 2003; Hurst 2005; Lewis 2006). Historically, territorial marking has received the vast majority of attention (see Gosling & Roberts 2001). In spotted hyenas, territorial maintenance has been the function of pasting behavior most emphasized as well (Gorman & Mills 1984; Henschel & Skinner 1991; Boydston et al. 2001). However, given that different age/sex classes of spotted hyenas paste at different rates (Mills & Gorman 1987), that hyenas frequently paste at locations other than territorial boundaries, including areas where intruders seldom appear (Hofer et al. 2001),

and that hyenas preferentially investigate the paste secretions of some donors more intensively than others (Drea et al. 2002), it is probable that pasting has intra-clan functions in addition to that of facilitating territorial maintenance. The functions served by scent marking can be best ascertained by determining the specific stimulus situations that elicit marking behavior, and the responses of animals to conspecific scents (Ralls 1971; Yahr 1983). In this chapter, we describe the general and specific contexts in which pasting is exhibited, and apply these data to predictions derived from competitive and reproductive functional hypotheses for spotted hyena pasting behavior.

First, we will determine whether pasting communicates dominance to conspecific competitors by systematically observing the pasting behavior of each adult hyena in a single clan over a two-year period, during which the hierarchical relationships among all adults remained stable. Previous research has indicated that adult immigrant males paste at higher rates than other members of hyena clans (Mills & Gorman 1987). Given that immigrant males are the lowest-ranking members of a clan, Mills & Gorman (1987) opined that the elevated pasting rates of males indicate that, contrary to most mammalian scent marking, pasting does not convey dominance status to competitors. However, before this assertion can properly be made, intra-sex hierarchical relationships need to be considered (Kappeler 1990; Woodmansee et al. 1991). Among immigrant males, high-ranking individuals may paste more frequently than low-ranking individuals, indicating that pasting likely is associated with social status.

Pasting by immigrant males may also serve as a form of sexual advertisement. A male's reproductive success is strongly dependent upon his length of tenure in the clan (Engh et al. 2002; Honer et al. 2007). Males do not typically sire cubs until they have

resided in a clan for at least two years (Engh et al. 2002; East et al. 2003). Since male hyenas are frequently targets of female aggression, pasting may allow newly tenured males to advertise their presence to females without suffering the harassment incurred by being in close proximity to females (Mills & Gorman 1987). This hypothesis does not require that newly arrived males paste at higher rates than males who have resided in a clan for a long time, but it does predict that males who invest in pasting early in their tenure should ultimately experience greater reproductive success than males who do not paste as often. We will test this hypothesis by determining whether a correlation exists between a male's pasting rate early in residency and the proportion of cubs he sires in the clan during his entire tenure.

Pasting by females could potentially function to advertise their reproductive state to males. In many mammals, females emit chemical cues that provide males with information about their reproductive condition (reviewed by Johnson 1973; Gorman & Trowbridge 1989). The benefits of advertising their impending sexual receptivity are likely greatest for females of solitary species wherein mates are difficult to locate and/or species in which females are dominant to, and aggressive toward, males, and consequently males are hesitant to approach females (e.g. Petrulis & Johnston 1997; Palagi et al. 2004). Additionally, in female-dominated species, there is little benefit associated with concealing ovulation, as creating paternity confusion is unnecessary for preventing male infanticide (Palagi et al. 2004). Female spotted hyenas are frequently aggressive towards adult male clanmates, and infanticide by male hyenas is a very rare event (East et al. 2003). Therefore, it is possible that female hyenas could benefit from advertising their impending ovulation to males. If female spotted hyenas do advertise

their sexual receptivity via scent marking than females should paste most often during periods when they are most likely to be receptive (i.e. late lactation period). This will be determined by systematically observing the scent marking behavior of adult female spotted hyenas across multiple reproductive cycles.

**Chapter Three**

As discussed above, the functions of scent marking are best ascertained by determining the stimulus conditions that elicit marking, and observing responses to marks by conspecifics (Yahr 1983). In the previous chapter we considered the contexts in which pasting occurred. In this chapter we consider the responses of spotted hyenas to conspecific scent. Definitive functional explanations for animal signals require knowing specifically what information is available in them (Drea et al. 2002). One of the primary means by which to determine the information content of scent marks is by conducting scent discrimination experiments. During these bioassays, subjects are presented with scents from donors that differ in only a single characteristic. If over multiple trials, utilizing multiple donors and subjects, one scent is consistently responded to in a different manner than other scents, then the scents are interpreted as being distinctive in some way and, therefore, capable of communicating information about the single characteristic to receivers. In an effort to determine the information content of paste, Drea et al. (2002) performed a series of scent bioassays with captive spotted hyenas, through which they demonstrated that paste contains information about the individual identity of the donor, as well as its sex. The authors suggest that these results indicate a potential

reproductive function for pasting behavior, a function previously believed to be inapplicable to spotted hyenas (Matthews 1939; Gorman & Mills 1984).

Muller-Schwarze (2001) has recommended that, if the logistic and experimental obstacles associated with field studies can be overcome, scent discrimination experiments should be conducted on free-ranging animals in natural environments. His reasoning was that the context for proper responses are likely to be encountered only in the field. Recently, others have echoed his sentiment (Drea et al. 2002; Begg et al. 2003). In this chapter, we will conduct scent discrimination experiments on three different clans of free-living spotted hyenas in the MMNR. The experiments will be staged primarily at communal den sites, where a majority of clan members congregate on a nightly basis. As hyenas are primarily nocturnal (Kruuk 1972; Kolowski et al. 2007), the bioassays will be conducted in the evening with the aid of night-vision binoculars. The paste samples that will be used in the experiments were obtained previously from the anal pouches of hyenas anaesthetized in the MMNR.

We have three specific objectives in performing these experiments. First, to discern whether hyenas discriminate between paste secretions from clanmates and non-clanmates, and if so, whether discrimination is due strictly to subjects' familiarity with donors, or whether donors from different clans have group-specific odors, as has previously been reported (Hofer et al. 2001; Burgener et al. 2006). Second, to verify that spotted hyenas are able to discriminate between paste samples based on the sex of donors. Third, to further pursue the suggestion by Drea et al. (2002) that pasting has a reproductive function, by determining whether paste from female donors contains information about their reproductive state. In concert with Chapter Two, these scent

discrimination experiments will provide us with a more comprehensive understanding of the adaptive significance of pasting in the spotted hyena.

**Chapter Four**

Parasitic and commensal organisms that reside on animal bodies have likely influenced behavioral evolution to a large extent (Jog & Watve 2005). Among these organisms are the bacteria associated with mammalian skin surfaces, which are widely believed to generate many of the volatile semiochemicals used by mammals during chemical communication (Albone et al. 1974; Leyden et al. 1981; Rennie et al. 1990; Parekh 2002; Alexy et al. 2003; Buesching et al. 2003; James et al. 2004; Voigt et al. 2005). One place these bacteria flourish is in mammalian scent organs (Albone 1984; Wyatt 2003). Most terrestrial members of the order Carnivora possess anal scent glands that provide warm, moist, anaerobic environments, ideal for the proliferation and growth of bacteria (Albone et al. 1978; Gorman & Trowbridge 1989). These scent glands also provide a substrate of lipids and proteins that are metabolized by the bacteria, generating volatile carboxylic acids that figure prominently in communication among carnivores (Gorman et al. 1974; Albone et al. 1978; Gorman & Trowbridge 1989). Through the production of carboxylic acids, commensal bacteria may provide carnivores with individual and/or group-specific odors (Gorman 1976). This explanation for the derivation of identity-specific odors in mammals is called the 'fermentation hypothesis' (Gorman 1976). All else being equal, if individual animals in a group harbor individually distinctive microbial populations in their scent glands, then their scent profiles will each be unique, and their scent marks will provide information to receivers about their

individual identity. Additionally, through frequent bodily contact and overmarking of one another's scent marks, members of the same social group can potentially come to share a common microbial population. Consequently, the scent profiles of members of the same social group will be similar and their scent marks will provide information to receivers about their group membership (Albone et al. 1974; Gorman 1976; Buesching et al. 2003). Group-specific odors potentially facilitate group cohesion and make cooperative defense of territories more efficient (Rasa 1973; Kruuk et al. 1984; Buesching et al. 2003).

In this chapter, we test the hypothesis that commensal bacteria are responsible for the production of group-specific odors in the spotted hyena (Burgener et al. 2008). Hyenas are highly gregarious, cooperatively territorial, large carnivores that have clan-distinctive scent profiles (Hofer et al. 2001; Burgener et al. 2008). If group-specific odors are the products of bacterial fermentation, then members of the same social group should have more similar bacterial populations in their scent glands than do members of different social groups. During a previous test of this prediction in the red fox, *Vulpes vulpes*, the prediction was not substantiated. However, the results in that study were likely biased by the differential recoverability of anaerobic bacterial forms in culture (Albone et al. 1978; Schloss & Handelsman 2004). We will avoid this potential bias by extracting bacterial 16S rDNA from paste secretions obtained from anaesthetized spotted hyenas, and performing recombinant cloning to generate a 'snapshot' of the microbial community of each individual hyena's anal pouch.

If group-specific odors in *Crocuta* are due to commensal bacteria, then after controlling for sex and reproductive state, clanmates should harbor more similar microbiota in their anal pouches than do hyenas from different clans. If we do find

differences between clans, further analysis of the generated clone libraries will allow us to describe specifically how bacterial populations differ among clans. If microbial differences between clans are not found, then we will have convincingly demonstrated that the bacterial mechanism for group-specific odors hypothesis is not applicable to spotted hyenas, as they come into frequent contact with conspecifics, and regularly overmark one another's scent marks.

Theis, K. R., A. L. Heckla, J. R. Verge, and K. E. Holekamp (2008). The ontogeny of pasting behavior in free-living spotted hyenas, *Crocuta crocuta*. In: Chemical Signals in Vertebrates 11 (Hurst, J.L., R.J. Beynon, S.C. Roberts, T.D. Wyatt, Eds.). Springer, New York. pp. 179-188.

CHAPTER ONE

# THE ONTOGENY OF PASTING BEHAVIOR IN FREE-LIVING SPOTTED HYENAS (*CROCUTA CROCUTA*)

## INTRODUCTION

An efficient communication system is a critical component of any animal society. Communication among members of a society can function to coordinate group activities, modulate intragroup aggression, and facilitate complex social interactions. In mammals, communication is often achieved via chemical signals that may convey information about the caller's reproductive state, social status, or individual, species and group identity (Wyatt 2003). One particularly common form of chemical communication among mammals is scent marking, which is the deliberate deposition of glandular secretions, urine or feces in the environment (Marler 1967; Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973). For many mammals, the temporal and spatial patterns of scent marking facilitate territorial maintenance and mate attraction via honest advertisement of resource holding potential (Rich & Hurst 1998; Gosling & Roberts 2001). However, it is becoming increasingly clear that the function of scent marking varies not only among species, but also among different age/sex classes within the same species (e.g. Drea et al. 2002; White et al. 2003). To date, few studies have addressed the ontogeny of scent marking in mammals (but see Rasa 1973; French & Cleveland 1984; Woodmansee et al. 1991; Palagi et al. 2002). The current study describes the ontogeny of anal gland scent marking ('pasting') in free-living spotted hyenas. Although some earlier studies have considered pasting in spotted hyenas (Kruuk 1972; Mills 1990; Woodmansee et al. 1991;

Boydston et al. 2001; Drea et al. 2002), we still do not have a complete understanding of its potential adaptive functions, particularly among juveniles.

Spotted hyenas are large, gregarious carnivores found throughout sub-Saharan Africa, living in clans of up to 90 individuals (Kruuk 1972). They are unique in that their societies are female-dominated, with adult immigrant males being lower-ranking socially than all natal animals. Natal animals inherit their mothers' rank positions within the clan's linear dominance hierarchy (Holekamp & Smale 1993; Engh et al. 2000), while immigrant males queue for social status amongst themselves (East & Hofer 2001). In addition to reasons associated with their unique biology, spotted hyenas are also intriguing subjects for communication studies because they emit a rich array of visual, vocal and chemical signals. Their most conspicuous chemical signaling behavior is pasting, wherein a hyena deposits anal gland secretions on grass stalks or, occasionally, on other objects in the environment (Kruuk 1972; Mills 1990). In the spotted hyena, all age/sex classes frequently 'paste' (Mills & Gorman 1987), although the pasting-like behaviors of many juveniles are likely ineffective as juvenile hyenas reportedly do not produce paste within their anal glands until they are 12-18 months old (Kruuk 1972). Juvenile pasting therefore presents a Darwinian puzzle (Alcock 2005), in that performing ineffectual behaviors typically reduces one's fitness.

The objectives of this study were: 1) to systematically determine the age at which spotted hyenas begin producing paste in their anal glands, 2) to determine how rates of juvenile pasting and overmarking vary during development, 3) to evaluate the effect of social rank on pasting behavior among juveniles, 4) to identify the immediate behavioral

contexts in which pasting occurs among juvenile spotted hyenas, and 5) to consider the potential functions of pasting by juvenile hyenas in light of this study's other findings.

## METHODS

This study was conducted in the Talek region of the Masai Mara National Reserve, Kenya. The subject population was a single large *Crocuta* clan that has been monitored continuously since 1988. Its members were identified by their unique spot patterns and other conspicuous characteristics, such as ear notches. Sex was determined from the dimorphic glans morphology of the erect phallus (Frank et al. 1990). Cubs were assigned birthdates by estimating their ages (± 7d) when first observed, based on their pelage and size. In this study, hyenas were considered cubs until they were one year old, and subadults from 13 to 30 months old. We further defined early, middle and late subadulthood as 13-18, 19-24 and 25-30 months respectively, in effort to draw comparisons with a previous study of captive juvenile hyenas (Woodmansee et al. 1991). Since juvenile hyenas 'inherit' their social ranks directly from their mothers (Engh et al. 2000), each subject was assigned its mother's relative rank within the clan at the time of the subject's birth.

Between 1994 and 2003, 113 different Talek hyenas were anaesthetized with Telazol (W.A. Butler Co.; 6.5 mg/kg) delivered from a $CO_2$-powered rifle (Telinject Inc.). To determine the age at which hyenas begin to produce paste, we examined the anal pouch of each hyena for the presence/absence of secretions. If a hyena was darted multiple times during this period, we only used its datum from the first darting. The ages of immigrant males were estimated from patterns of tooth wear using methods described

by Van Horn et al. (2003). To evaluate the effects of age and sex on the likelihood of paste production by the anal glands we employed multiple logistic regression (SAS v8.2, 2001). Throughout this study, results were considered statistically significant when $P < 0.05$ (two-sided). All descriptive statistics have been presented as mean ± standard error.

In the current study we also utilized archived behavioral observation data gathered between 1988 and 1996. We collected repeated behavioral measures from 14 male and 12 female hyenas, from birth through 30 months of age. Behavioral observations were made at dens, kills and elsewhere. During observations, scent marking, agonistic interactions, and greeting behaviors were recorded via all occurrence sampling (Altmann 1974). Rates of pasting and overmarking were calculated as the number of events per individual per hour observed (Woodmansee et al. 1991). Since scent marking rate data were not normally distributed, comparisons were made from these data using nonparametric Friedman ANOVA, Mann-Whitney U (Statistica v6.1, 2002), and Wilcoxon signed-rank tests (SAS).

Each pasting event was assigned a context based upon the behavioral and social events occurring in the two minutes preceding the pasting behavior. If a hyena pasted on grass stalks, etc. that had been previously scent marked by others in the same observation session, the context was 'overmarking' (om). If a hyena pasted in a spot not previously scent marked by others in the observation session, yet just after another hyena had pasted elsewhere, the context was ' adjacent marking' (am). If a hyena pasted following a submissive and/or aggressive interaction with a social partner, or following a significant, high-intensity aggressive interaction between group-mates in the same observation session, the context was 'agonism' (agon). If a hyena pasted after greeting with another

hyena (i.e. mutual sniffing of the anogenital region), or after offering or receiving an unrequited greeting invitation (i.e. one hyena lifts its hindleg thereby conspicuously exposing its anogenital region to a second hyena that does not subsequently sniff the region), the context was 'greeting' (grt). If pasting was preceded by more rare stimuli, such as participation in border patrols, inter-clan conflicts or allomarking of hyena cubs, the context was categorized as 'other' (oth). If more than one of these contexts preceded a paste event, we assigned the context that most immediately preceded the paste event. Lastly, if a pasting event was not preceded within two minutes by evident stimuli, the context was 'spontaneous' (spon). Paste events that were not preceded by two complete minutes of all-occurrence sampling were labeled 'unknown.' For each hyena, we calculated the proportion of pasting events that occurred in each of these contexts. Proportion data were arcsin square root transformed and comparisons were made using paired and unpaired t-tests (Statistica). We employed the Holm's sequentially-rejective Bonferroni method to evaluate the statistical significance of multiple comparisons (Shaffer 1995).

## RESULTS

Anaesthetized hyenas ranged from 7 to 143 months of age. Investigation of their anal pouch contents revealed a highly significant effect of age (Wald $\chi^2 = 16.85$, $P < 0.0001$), but not of sex (Wald $\chi^2 = 0.007$, $P = 0.93$), on the likelihood of paste production. It appears that spotted hyenas do not consistently produce paste until their third year of life (Figure 1.1). The youngest age at which secretions were observed within an anal

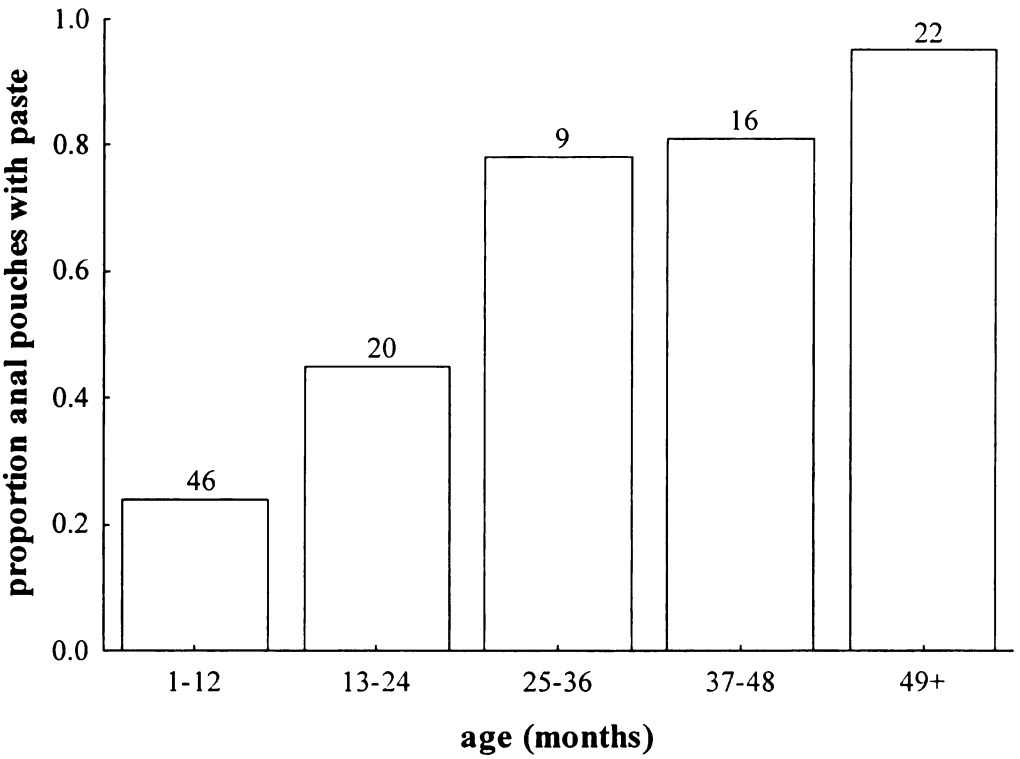



Figure 1.1. Effect of age on paste production in spotted hyenas. Numbers above the bars indicate the number of hyenas sampled in each age class.

pouch was 9 months, although paste production at this age is uncommon, as only three of the 12 cubs darted in their ninth month had appreciable secretions in their anal pouch. Eight cubs were darted prior to nine months of age, and all their pouches appeared devoid of secretions. In this study, the sex, social rank and body mass of cubs did not appear to affect paste production (unpublished data), however, these factors should be considered in a more controlled manner, with larger sample sizes, before firm conclusions are drawn.

For the behavioral portion of this study, we observed each of the 26 juvenile hyenas for 98 ± 7 hrs (range: 37 – 165). Collectively, they exhibited 1491 paste events, for an average of 57 ± 7 pastings per hyena (range: 8 – 138). Among both males and females, cub and subadult pasting rates were similar (Wilcoxon signed-rank test; $N_m$= 14, S = -16.5, P = 0.33; $N_f$= 12, S = 14, P = 0.30). Additionally, neither males nor females varied their pasting rates among early, middle and late subadult periods (Friedman ANOVA; $N_m$= 8, $\chi^2$ = 1.75, P = 0.42; $N_f$= 12, $\chi^2$ = 3.87, P = 0.14; analyses of individuals observed > 3 hr in each period). Although there was no effect of age, males pasted more frequently than females as cubs (Mann-Whitney U test, U = 42, P = 0.03; Figure 1.2), but not as subadults (U = 76, P = 0.71). Pasting rates were not influenced by social rank in any sex/age class examined here (male cub: $R^2$ = 0.133, $F_{1,12}$ = 1.85, P = 0.2; female cub: $R^2$ < 0.001, $F_{1,10}$ < 0.01, P = 0.98; male subadult: $R^2$ = 0.082, $F_{1,12}$ = 1.07, P = 0.32; female subadult: $R^2$ = 0.073, $F_{1,10}$ = 0.79, P = 0.4).

Males overmarked more when they were subadults than cubs (Wilcoxon signed – rank test, S = 32.5, P = 0.04; Figure 1.3). Males did not, however, vary the proportional abundance of overmarking across early, middle and late subadult periods (Friedman

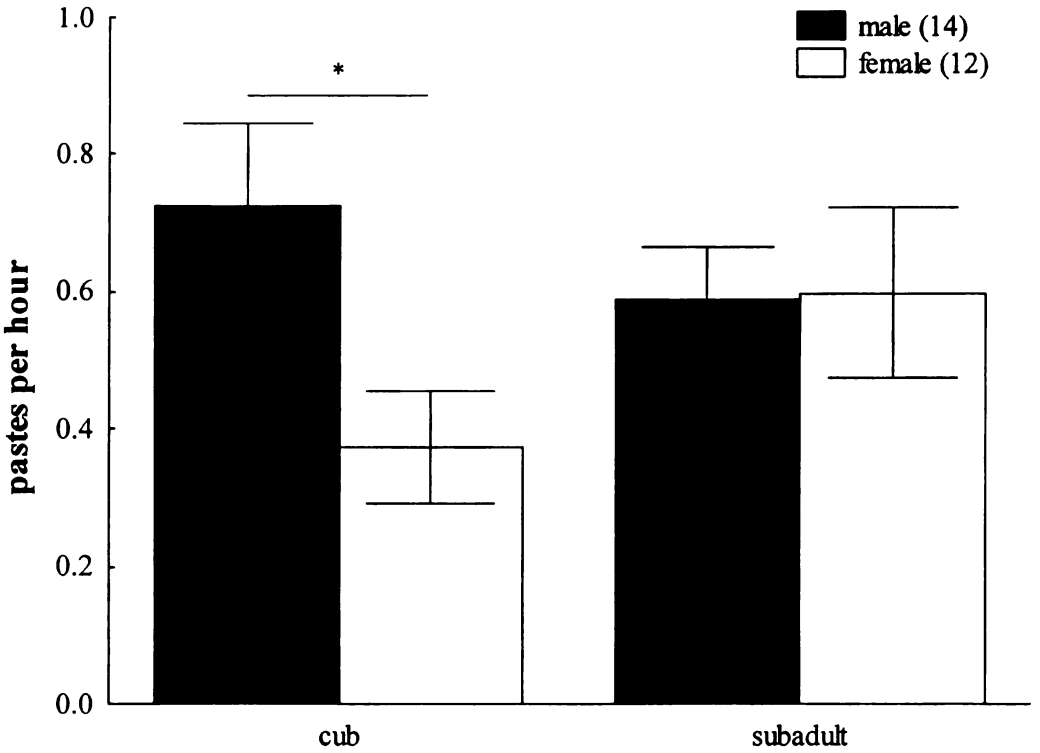



Figure 1.2. Hourly pasting rates of juvenile male and female spotted hyenas by age. The asterisk indicates a statistically significant difference ($P < 0.05$). Numbers in parentheses indicate samples sizes.

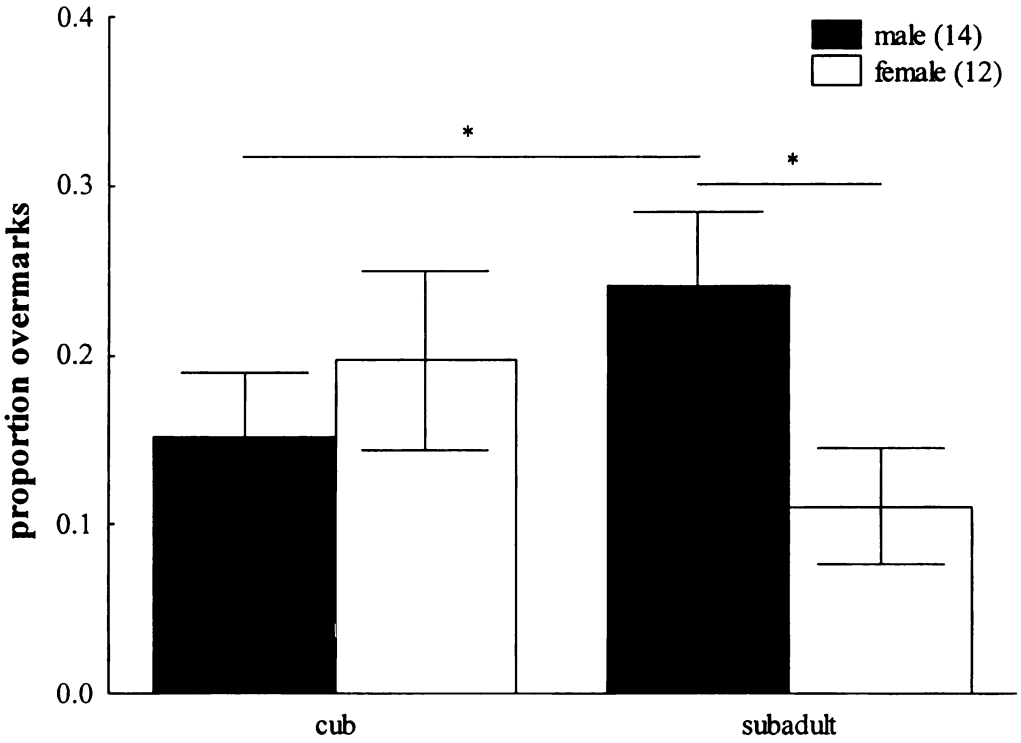



Figure 1.3. Proportion of spotted hyena pasting events that were overmarks of conspecific scent marks, by age and sex. Asterisks indicate statistically significant differences ($P < 0.05$). Numbers in parentheses indicate sample sizes.

ANOVA, N = 6, $\chi^2 = 3.0$, P = 0.22). Female overmarking did not vary with age (cub vs. subadult: S = -15.5, P = 0.24; across subadult periods: N = 6, Friedman ANOVA $\chi^2 = 3.5$, P = 0.17). Although cubs did not overmark disproportionately by sex (Mann-Whitney U test, U = 75, P = 0.67), subadult males overmarked more than females (U = 37, P = 0.02). Social rank did not influence the extent to which hyenas overmarked in any age/sex class (male cub: $R^2 = 0.21$, $F_{1,12} = 3.14$, P = 0.1; female cub: $R^2 < 0.001$, $F_{1,10} < 0.01$, P = 0.97; male subadult: $R^2 < 0.001$, $F_{1,12} < 0.01$, P = 1.0; female subadult: $R^2 = 0.07$, $F_{1,10} = 0.71$, P = 0.42).

In general, the primary contexts of pasting for all age/sex classes in this study were spontaneous, overmarking, adjacent marking, and following agonistic interactions (Figure 1.4A,B). Among males, the proportion of pasting events occurring spontaneously and in the context of overmarking varied between cub and subadult periods (paired t-test, N = 9; spon: t = 3.49, P = 0.01; om: t = -2.4, P = P = 0.04; males pasting > 10 times each category; Figure 1.4A,B), with male subadults exhibiting decreased spontaneous pasting behavior and increased overmarking. Males did not vary their propensity for pasting in other contexts between cub and subadult age periods (am: t = -0.09, P = 0.93; agon: t = -1.46, P = 0.18; grt: t = -0.4, P = 0.7). Female hyenas did not vary the proportion of pasting events occurring in each behavioral context between cub and subadult periods (N = 6; spon: t = -0.49, P = 0.64; om: t = 0.71, P = 0.51; am: t = 0.61, P = 0.57; agon: t = -1.41, P = 0.22; grt: t = 0.09, P = 0.94; Figure 1.4A,B).

Among cubs, there were no significant differences between males and females in the behavioral contexts preceding pasting behavior (t-test; spon: t = 0.39, P = 0.7; om: t =

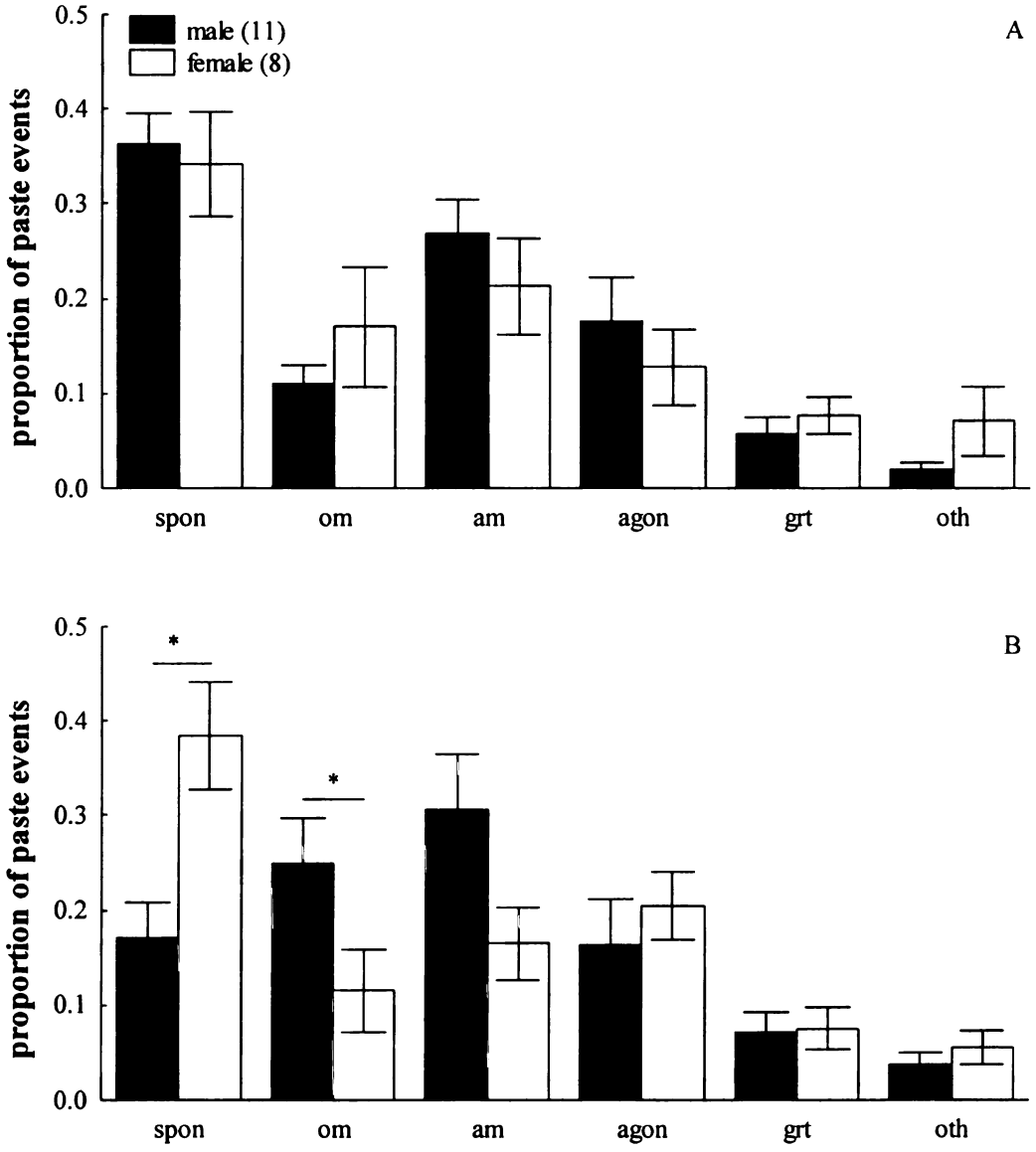



Figure 1.4. Behavioral contexts of pasting by A) cubs and B) subadults. Approximately one percent of paste events were assigned an 'unknown' context. These events were excluded from all analyses. Asterisks indicate statistically significant differences after Holm's sequentially-rejective Bonferroni method was employed. Numbers in parentheses indicate sample sizes.

-0.24, P = 0.81; am: t = 0.77, P = 0.45; agon: t = 0.84, P = 0.41; grt: t = -0.73, P = 0.47; Figure 1.4A). However, sex did significantly affect pasting context among subadults, with females pasting spontaneously more often than males (t = -2.89, P = 0.01), and males overmarking more often than females (t = 2.39, P = 0.03; Figure 1.4B). Male and female subadults did not vary in the degree to which they pasted in other contexts (am: t = 1.26, P = 0.23; agon: t = -1.03, P = 0.32; grt: t = -0.28, P = 0.78).

## DISCUSSION

Although a few individuals in our study population produced paste as early as nine months, it appears that spotted hyenas do not consistently generate anal gland secretions until their third year of life. This is when both males and females mature sexually (Matthews 1939), with males beginning to disperse from their natal clans (Frank et al. 1995; Smale et al. 1997), and many females conceiving their first litters (Holekamp et al. 1996). It is also during this period that juveniles in our study began to participate in territorial defense (see also Boydston et al. 2001). Therefore, it appears that paste production in spotted hyenas is similar to exocrine scent gland activity in many mammals, in that it seemingly coincides with the appearance of adult typical behaviors (Yahr 1983). Interestingly, although consistent paste production appeared to coincide with reproductive maturity, this did not lead to an increased frequency of scent marking activity. Our finding contrasts with that of Woodmansee et al. (1991), who found that captive spotted hyenas reared in peer-only groups greatly increased their frequency of pasting late in their second year of life. The difference between their study and ours

reaffirms the influence of social and environmental contexts on mammalian scent marking behavior (French & Cleveland 1984; Muller-Schwarze 2001).

In this longitudinal study of 26 individuals, the frequency of 'pasting' by juvenile spotted hyenas was consistently high throughout early development. Furthermore, we have confirmed that cubs engage in this behavior despite their not producing anal gland secretions (Kruuk 1972). With greater emphasis being placed in recent studies on understanding selection during juvenile life stages (Holekamp & Smale 1998; Altmann & Altmann 2003), this seemingly ineffectual behavior presents an intriguing Darwinian puzzle (Alcock 2005). Although pasting by juvenile hyenas does not appear to bear substantial costs (e.g. time, energy, social repercussion), and although juvenile pasting need not necessarily be an adaptive trait (Gould & Lewontin 1979), given the high frequency at which young hyenas pasted in this study, functional hypotheses should be considered.

**Results in relation to possible adaptive functions of pasting by juvenile hyenas**

One adaptive hypothesis suggests that, rather than depositing scents in the environment, cubs are acquiring group-specific odors that facilitate conspecific recognition of them as members of the group before they are widely known as individuals. Rasa (1973) proposed that mature African dwarf mongooses (*Helogale undulate rufula*) communally allomark newborns to ensure that they are recognized by conspecifics as mongooses rather than prey. Interestingly, in the current study, we also observed multiple instances of mature animals allomarking young cubs (unpublished data). For spotted hyena cubs, overmarking conspecific paste may be a mechanism for

actively donning group-specific odors. Among social animals, being effectively recognized as a member of a group can significantly reduce the amount of aggression an individual receives from its social partners (e.g. Hurst et al. 1993). Being recognized as a group member may be especially important for young carnivores because they have not yet developed the morphological weapons or behavioral repertoire necessary for defending themselves against conspecifics, particularly in species that exhibit conspecific infanticide, such as spotted hyenas (Hershel & Skinner 1991; Frank et al. 1995; personal observation). There have been numerous reports of other carnivores rubbing their cheeks, necks and backs against conspecific scent marks (Reiger 1979). For example, European badger (*Meles meles*) cubs occasionally rub themselves against the extruded scent glands of adult conspecifics that are engaged in scent marking (Buesching et al. 2003), and meerkats (*Suricata suricatta*) frequently rub their bodies against communal scent posts (Moran & Sorensen 1986). In both species, the rubbing behavior is believed to result in the actors picking up, rather than depositing, group-specific scents (Buesching et al. 2003; Moran & Sorensen 1986).

A second functional hypothesis to explain pasting by hyena cubs is that it exposes them to symbiotic bacteria that are potentially necessary for paste production in general, and for the generation of group-specific odors in particular. In bobtail squid (*Euprymna scolopes*), bacterial colonization is a prerequisite for normal development of the luminescent organ in juveniles (McFall-Ngai & Ruby 1991). The rudimentary organs of juvenile squid have ciliated projections that facilitate bacterial inoculation. Once inoculation has occurred, the ciliated structures regress (McFall-Ngai & Ruby 1991). We see a rough analogy between these ciliated structures and overmarking of conspecific

paste by spotted hyena cubs. In mammals, bacteria are likely responsible for generating many of the semiochemicals emanating from scent glands (Albone 1984). One mechanistic hypothesis to explain the generation and maintenance of mammalian group-specific odors is that members of a social group, through bodily contact and overmarking, come to share a common microbial population in their scent glands (Albone et al. 1974; Gorman 1976). Provided they exist on similar organic substrates, shared microbial communities should theoretically produce shared volatile odorants as a by-product of their metabolism. Pasting therefore potentially provides both short and long-term group-recognition benefits to hyena cubs, by facilitating their acquisition of both group-specific odors and bacteria.

We found that male cubs pasted more frequently than their female peers, suggesting that this sexually dimorphic behavior might be mediated by differential exposure of males and females to specific gonadal steroid hormones during development (Yahr 1983). Given that most cubs did not produce paste secretions, and that male and female cubs did not significantly differ with respect to the behavioral contexts in which they pasted, it is doubtful that the functional explanations for pasting behavior differ between male and female cubs. Although male and female subadults did not paste at different rates (see also Mills & Gorman 1987; Woodmansee et al. 1991), they did paste under different behavioral and social circumstances. Subadult females pasted spontaneously twice as often as males did, whereas subadult males overmarked conspecific scent marks far more frequently than did females. These sex differences in context suggest that the functions of pasting likely differ between subadult females and males. Spontaneous scent marking is typically viewed as a form of indiscriminate self-

advertisement wherein the signaler communicates information about its identity and presence to members of its social group as well as its neighbors (Wolff et al. 2002). Self-advertisement may be particularly beneficial for subadult female spotted hyenas as females in this species are philopatric (Smale et al. 1997). For female *Crocuta*, establishing and reinforcing their individual identity, group membership, and position within the clan's linear dominance hierarchy may facilitate their access to contested resources and ultimately their reproductive success (Holekamp & Smale 1993; Smale et al. 1993; Holekamp et al. 1996; Holekamp & Smale 1998). It is important to note that self-advertisement would benefit all subadult females regardless of social rank and that, in the current study, female scent marking activity was not influenced by social rank.

It is less apparent why, in an adaptive sense, subadult males overmark clanmates' scents more often than do subadult females. Scent marking directly on top of, or immediately beside, the previously deposited scent marks of conspecifics is typically perceived as an honest and effective means by which to advertise one's competitive ability to both competitors and prospective mates (reviewed by Gosling & Roberts 2001; Hurst & Beynon 2004; Johnston 2005; Ferkin & Pierce 2007). However, at least among subadult male hyenas this does not seem to be the case. In the current study, subadult males directed three-fourths of their overmarks toward other subadult male and adult male clanmates (unpublished data), suggesting that subadult males may be targeting their competitors for overmarking. We also found that males overmarked conspecifics significantly more as subadults than cubs, and that subadult males exhibited a tendency to increase overmarking activity as they matured (unpublished data). However, we did not find any correlation between overmarking activity and social rank. Therefore, a

competitive function for subadult male overmarking behavior appears unlikely. Of course, a confounding factor here is that we cannot be certain which individuals were producing secretions in their anal glands and, therefore, were capable of actually depositing scent marks upon others' marks when overmarking. Ideally, an analysis assessing the degree of correlation between overmarking and social rank would include only individuals that were already producing paste. However, even for subadult males that have begun to produce both paste in their anal glands and sperm in their testes, overmarking is unlikely to make them more competitive for reproductive opportunities because male hyenas seldom sire offspring in their natal clans (Engh et al. 2002; Van Horn et al. 2008).

Although overmarking by subadult male hyenas is unlikely to function in advertising dominance or attracting mates, given the high degree of sociality in *Crocuta*, overmarking by subadult males may function to reinforce their group membership, thereby facilitating social cohesion. Spotted hyena clans are fission-fusion societies in which subgroups change frequently in size and composition (Kruuk 1972; Smith et al. 2007). Frequently, when reuniting after being separated, members of hyena clans engage in conspicuous greeting behaviors, suggesting that the maintenance of social bonds is very important in this species (East et al. 1993). Additionally, extrusion of the anal pouch during greeting is most common among individuals who meet only infrequently (East et al. 1993; Hofer et al. 2001). As subadult males approach their third year of life they begin the process of natal dispersal (Smale et al. 1997). The dispersal process entails multiple exploratory forays into foreign territories, resulting in subadult males becomingly increasingly absent from their natal clanmates for extended periods of time (Smale et al.

1997). It is conceivable that the heightened overmarking activity of subadult males effectively compensates for their infrequent social interaction with clanmates as a result of their dispersal forays, and facilitates their being non-aggressively received upon their return (see Hurst et al. 1993; Rostain et al. 2004). A problem that must be resolved here, however, is why males increase overmarking activity, but not self-advertising spontaneous pasting behavior, when they become subadults. Resolution may lie in the mechanisms underlying scent deposition and information transfer in this species.

**Results in relation to mechanistic considerations**

When animals deposit scent marks upon those of conspecifics, three mechanistic results are possible (Johnston et al. 1994). First, the top mark may entirely mask the bottom mark. Second, the top and bottom marks may remain distinct and individually identifiable. Third, the top and bottom marks may blend, forming a new, composite scent. Scent-masking is typically attributed to solitary species and is believed to function in concealing the scent marks of competitors and/or in mate-guarding (Johnston et al. 1994; Ferkin & Pierce 2007). In the current study of juvenile hyenas, overmarking behavior was most common among subadult males. The heightened frequency of overmarking by subadult male hyenas is not consistent with the scent-masking hypothesis because overmarking by subadult males was not correlated with social status, male hyenas seldom sire cubs in their natal territories (Engh et al. 2002), and mate-guarding is not an effective reproductive strategy for males in this species (East et al. 2003; Szykman et al. 2007).

If the top and bottom scent marks remain distinct and individually identifiable following overmarking, then a chemical bulletin board is generated (Johnston et al.

1994). Chemical bulletin boards are assumed to occur among wide-ranging mammalian species, as they permit each participant to advertise their identity and presence to all receivers that happen by (Johnston et al. 1994; Ferkin & Pierce 2007). Consequently, contributing to bulletin boards is an effective means of self-advertisement, perhaps even more so than scent marking spontaneously. Given the aforementioned value of self-advertisement for subadult females, if overmarking by spotted hyenas generates a chemical bulletin board, then subadult females should overmark clanmates as frequently, if not more often, than subadult males. We have shown this is not the case.

Scent-blending through overmarking is believed to occur among colonial species, as the consequent composite, group-specific odor permits rapid and efficient recognition of group-mates (Johnston et al. 1994; Ferkin & Pierce 2007). We have already hypothesized that spotted hyena cubs overmark clanmates to acquire group-specific odors that facilitate their being recognized as members of their social group, and perhaps also to acquire group-specific bacterial communities responsible for the production of clan-specific odors. We further postulate that subadult male hyenas, by frequently contributing their scent to the composite odor of the clan (see Mills & Gorman 1987), simultaneously reinforce their group membership and ensure their possession of group-specific bacterial communities. Either subadult females can accomplish these same objectives by overmarking less often, perhaps as a result of their continued presence in the clan's territory, or the importance of advertising individual identity supercedes communicating group membership for subadult female hyenas.

**Conclusions**

In this study, we have proposed multiple hypotheses for pasting behavior in male and female juvenile hyenas. Although most spotted hyena cubs do not produce appreciable quantities of paste in their anal scent glands, they still frequently engage in pasting behavior. We here suggest that hyena cubs are picking up, rather than depositing, scents. As spotted hyena paste secretions bear clan-specific chemical signatures (Hofer et al. 2001), cubs may be donning clan-specific odors that facilitate their being recognized as members of the group before they are widely known as individuals. Furthermore, by engaging in pasting behavior, cubs may be acquiring symbiotic bacteria that potentially figure prominently in producing the odorants associated with paste secretions.

Among subadults, paste production is much more common, as approximately two-thirds of all subadult spotted hyenas investigated in this study had perceptible amounts of paste in their anal pouches. Given the high incidence of spontaneous pasting behavior among subadult female hyenas, the current study suggests that pasting may serve as a form of self-advertisement for them. Among subadult males, however, spontaneous pasting was relatively infrequent, while overmarking of clanmates' scent marks was common. Whereas in most mammals, overmarking is believed to serve competitive and reproductive functions (Johnston 2005; Ferkin & Pierce 2007), our data suggest that, at least among subadult male spotted hyenas, overmarking may reinforce the signaler's group membership.

We emphasize that each of these hypotheses is speculative and that collectively they do not represent an exhaustive list of the potential adaptive functions of scent marking by juvenile spotted hyenas. A line of further inquiry that appears most necessary

is an investigation of the bacterial communities of spotted hyena anal pouches. A preliminary study revealed clan-specific differences in the volatile odorants emanating from paste secretions (Hofer et al. 2001). Although a bacterial mechanism for these differences has been proposed (Burgener et al. 2006), this hypothesis has not yet been tested. In the future, we will determine the degree of variability in the bacterial communities among spotted hyena anal pouches, and, if variability exists, whether it is clan-specific. If it is established that the bacterial communities in *Crocuta* anal pouches are clan-specific, then the hypotheses introduced in the current study will have received considerable support.

CHAPTER TWO

# INTRAGROUP FUNCTIONS OF SCENT MARKING BY ADULT SPOTTED HYENAS (*CROCUTA CROCUTA*)

## INTRODUCTION

Scent marking, defined as the deliberate deposition of urine, feces, and/or the products of exocrine scent glands in the environment, is a common form of communication among mammals (Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973). The information potentially communicated via mammalian scent marking includes the donor's species, group, sex, age and individual identity, as well as its reproductive, social, nutritional and health status (Ferkin et al. 1997; Gosling & Roberts 2001; Zala et al. 2004; Hurst 2005). In general, mammals signal this information to achieve three broad functional objectives: 1) territory and resource defense, 2) maintenance of dominance and social relationships, and 3) mate attraction and reproductive synchrony (Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973; Gosling 1982; Rich & Hurst 1998; Heymann 2000).

Although historically, emphasis has been placed on the fitness benefits accrued by adult males scent marking in territorial contexts (Gosling & Roberts 2001; Hurst & Beynon 2004), recent studies have demonstrated that the functions of scent marking vary widely, not only among mammalian species, but also among different age, sex, reproductive and social classes within a single species (e.g. Begg et al. 2003; Miller et al. 2003; Swaisgood et al. 2004; Hurst 2005; Lewis 2006; Jordan 2007). Additionally, it has become increasingly clear that we need to develop a more comprehensive understanding of the functions of scent marking in highly gregarious species (Scordato & Drea 2007;

Muller & Manser 2008), especially given that social complexity potentially drives signaling complexity (e.g. Freeberg 2006). In this study, we evaluated potential functions for scent marking in a highly gregarious carnivore, the spotted hyena, *Crocuta crocuta*.

Spotted hyenas live in complex social groups, called clans, that typically contain 40 - 80 individuals (Kruuk 1972; Trinkel et al. 2007). Members of each clan cooperatively defend their group's territory from neighboring hyenas, and they also defend ungulate kills from both other hyenas and African lions, *Panthera leo* (Kruuk 1972; Mills 1990; Henschel & Skinner 1991; Boydston et al. 2001). Hyena clans contain multiple breeding males and multiple overlapping generations of females. Clans are structured by rigid linear dominance hierarchies (Frank 1986a,b). In contrast to most mammalian social systems, a hyena's position within its clan's dominance hierarchy is not determined by its fighting ability. Instead, natal hyenas 'inherit' the social ranks of their mothers (Frank 1986b; Holekamp & Smale 1991, 1993; Smale et al. 1993; Engh et al. 2000), and adult immigrant males, all of whom are subordinate to natal animals, queue for dominance status with other immigrant males (Smale et al. 1997; East & Hofer 2001).

In addition to possessing a rich repertoire of vocal and visual signals, spotted hyenas communicate chemically through greeting ceremonies, scent-rubbing, forepaw scratching, defecating at latrines, and anal gland "pasting" (Kruuk 1972; Bearder & Randall 1978; Mills 1990; Henschel & Skinner 1991; East et al. 1993). Anal gland pasting is a common and conspicuous form of scent marking wherein hyenas drag their extruded anal pouches over grass stalks and, in doing so, typically deposit a thin layer of anal gland secretion near the tops of the stalks (Matthews 1939; Kruuk 1972; Mills 1990). Previous investigations of pasting behavior among spotted hyenas have shown that

it figures prominently in territorial maintenance (Kruuk 1972; Mills & Gorman 1987; Henschel & Skinner 1991; Boydston et al. 2001). Indeed, some have suggested that pasting functions exclusively in territorial maintenance for *Crocuta* (Gorman & Mills 1984). However, this seems unlikely given that all age/sex classes of spotted hyenas paste (Mills & Gorman 1987; Woodmansee et al. 1991; Theis et al. 2008), and that they often do so, even in areas where hyena population density is high, at locations other than territorial boundaries, such as communal dens where intruders are typically not seen (Hofer et al. 2001; Theis et al. 2008). Furthermore, spotted hyenas have been observed pasting in a wide variety of social and behavioral contexts (Kruuk 1972; Mills & Gorman 1987; Woodmansee et al. 1991; Theis et al. 2008). Although others have suggested that pasting has intragroup functions for adult spotted hyenas (Mills & Gorman 1987; Woodmansee et al. 1991; East et al. 1993; Hofer et al. 2001; Drea et al. 2002), no one has yet systematically evaluated these hypotheses. Therefore, the objectives of our study were to document the circumstances under which pasting occurs, and to evaluate four hypotheses suggesting intragroup functions for pasting behavior by adult spotted hyenas (Table 2.1). This was achieved by evaluating pasting rates and determining the broad and specific socio-ecological contexts in which pasting occurs among free-living hyenas. Determining the contexts in which scent marking occurs among free-living animals has been deemed critical in ascertaining its functional significance (Muller-Schwarze 2001; Thomas & Kaczmarek 2002; Begg et al. 2003).

The first hypothesis we evaluated ($H_1$ in Table 2.1) suggests that pasting functions to advertise dominance status to competitors (Woodmansee et al. 1991). Scent

Table 2.1 Intragroup functions of pasting by adult spotted hyenas: hypotheses and predictions. Hypotheses are neither exhaustive nor mutually exclusive. Italicized letters in parentheses indicate the data set used to test each prediction. The composition of each data set is explained in the Methods section.

| Hypothesis | Prediction | Data Set |
|---|---|---|
| $H_1$: Advertise dominance status | | |
| | $P_1$: positive correlation between social rank and pasting rate | (*B*) |
| | $P_2$: pasting rates highest in vicinity of competitors | (*A*) |
| $H_2$: Facilitate social cohesion | | |
| | $P_1$: pasting rates higher after absence from clan than before | (*C*) |
| $H_3$: Advertise female sexual receptivity | | |
| | $P_1$: pasting rates highest when likelihood of fertility is greatest | (*D*) |
| | $P_2$: during fertile periods, pasting rates highest in vicinity of males | (*D*) |
| $H_4$: Advertise male availability for reproductive opportunities | | |
| | $P_1$: positive correlation between pasting rate early in tenure and relative reproductive success | (*A*) |

marking has been shown to be an honest indicator of competitive ability in many mammals (Ralls 1971; Gosling & Roberts 2001; Rich & Hurst 1998, 1999). If pasting has a competitive function among spotted hyenas, then we expected to observe a strong positive correlation between social status and pasting frequency (Ralls 1971). It was previously suggested that pasting does not communicate dominance status to competitors because adult immigrant male hyenas seemingly engage in the behavior more frequently than other members of hyena clans, and they are also the lowest-ranking members of their respective clans (Mills & Gorman 1987). However, given that all adult female hyenas are higher-ranking socially than all adult immigrant males, dominance should be considered separately for each sex when determining the effect of social status on scent marking behavior (Kappeler 1990; Woodmansee et al. 1991). Therefore, in this study, we evaluated the dominance hypothesis separately for males and females. We additionally tested the prediction that, if pasting advertises dominance status, then rates of pasting should be highest when hyenas are in the vicinity of their intragroup rivals (Macdonald 1985; Mech et al. 2003).

The second hypothesis we assessed suggests that pasting functions to facilitate social cohesion within clans by reaffirming group membership (East et al. 1993). In house mice, it has been shown that scent marking plays a critical role in maintaining familiarity and tolerance among group members (Hurst et al. 1993). If pasting serves this purpose in adult spotted hyenas, then individuals who are absent from their clans for extended periods of time should paste more frequently following their return than they did prior to their departure (East et al. 1993).

The third hypothesis suggests that pasting functions to advertise female sexual receptivity. In many mammals, females emit chemical cues that provide males with information about their reproductive condition and receptivity (Johnson 1973; Johansson & Jones 2007). The benefits of advertising sexual receptivity are likely greatest for females of asocial species in which mates are difficult to locate, and in species in which females are dominant to, and aggressive toward, males such that males are hesitant to approach females (Petrulis & Johnston 1997; Palagi et al. 2004). Female spotted hyenas are indeed frequently aggressive toward male conspecifics (Frank 1986b; Szykman et al. 2003; East et al. 2003), so female hyenas could potentially benefit from advertising their impending ovulation to males. Furthermore, male spotted hyenas associate most closely with females when they are fertile (Szykman et al. 2001), so information about ovulation and female sexual receptivity appears to exist in this species. This information is potentially communicated via paste. Paste secretions do contain information on donor sex, and it has been suggested that they contain information on female reproductive state as well (Drea et al. 2002). If female spotted hyenas advertise their sexual receptivity via pasting, then they should paste most frequently during periods when they are most likely to be fertile: during late lactation. This is the period during which females typically wean their latest litters and become pregnant again (Szykman et al. 2003). Additionally, during fertile periods, female hyenas should paste most frequently when in the presence of prospective mates.

The fourth hypothesis suggests that pasting by adult males functions to advertise their presence in the clan, and thereby their availability to females for reproductive opportunities (Mills & Gorman 1987). Male hyenas seldom sire offspring in their natal

clans and most males disperse (Smale et al. 1997; Van Horn et al. 2008). Among adult immigrant males, reproductive success is largely dependent upon a male's length of tenure in his current clan (Engh et al. 2002; East et al. 2003). Most immigrant males do not successfully sire cubs until they have resided in a clan for at least two years, and most progeny appear to be sired by males with at least four years of tenure (Engh et al. 2002; East et al. 2003). As females exercise an unusual degree of mate choice in this species (Honer et al. 2007), and as females appear to be selecting mates that have been immigrants in their clans for several years (Engh et al. 2002; East et al. 2003), males could seemingly benefit from advertising their presence in their new clans early and often. Since male hyenas are frequent targets of female aggression (Frank 1986b; Szykman et al. 2003; East et al. 2003), pasting may afford newly tenured males the opportunity to advertise their presence to females without suffering the harassment incurred by being in close physical proximity to them (Mills & Gorman 1987). This hypothesis does not require that newly arrived males paste at higher rates than males who have resided in a clan for a long time, but it does predict that males who invest in pasting early in their tenures should ultimately experience greater reproductive success than males who do not do so. Specifically, among immigrant males, there should be a positive correlation between males' pasting rates early in their residencies and the proportional abundances of cubs they sire during their tenures.

## METHODS

### Study area

We have been intensively studying a single clan of spotted hyenas in the Talek region of the Masai Mara National Reserve, Kenya, since May, 1988. This region consists of rolling grassland, scattered brushland, and narrow stretches of riparian forest. The Reserve supports sizable concentrations of resident ungulates (Thomson's gazelle, *Gazella thomsonii*; topi, *Damiscilus korrigum*; impala, *Aepyceros melampus*), as well as large migratory herds of wildebeest (*Connochaetes taurinus*) and zebra (*Equus burchelli*) that are present with the resident antelope from approximately June through September each year (Frank 1986a; Holekamp et al. 1999; Cooper et al. 1999). As part of our long-term study of the Talek clan, we have conducted bi-monthly prey censuses within the clan's home range, as described in Holekamp et al. (1999). In the current study, we averaged the total prey counts of censuses occurring in each month of the study, and characterized each month as having high (474 – 3068 head; upper third of monthly values), medium (264 – 473, middle third), or low (34 – 263, lower third) prey abundance.

There are typically two rainy seasons in the Reserve, one from March – May, and another from November – December, although inter-annual variation exists (Darling 1960; Holekamp et al. 1999). As part of our long-term monitoring efforts we recorded rainfall levels (mm) daily from a rain gauge located within the Talek clan's home range. In the current study, we summed the daily rainfall measures within each month of the study, and characterized each month as having high (105 – 336 mm, upper third of

monthly values), medium (47.1 – 104.9, middle third), or low (3 – 47, lower third) rainfall volumes.

**Study subjects**

The current study uses systematic behavioral data collected daily via direct observations of Talek hyenas between September, 1988 and April, 2000. During that time the Talek clan maintained a territory of approximately 65 $km^2$, and was comprised of 20 – 23 breeding females, 10 – 12 adult immigrant males, and 30 – 40 offspring (Holekamp et al. 1999). Members of the clan were identified by their unique spot patterns and other conspicuous physical characteristics. Sex was determined from the dimorphic glans morphology of the erect phallus (Frank et al. 1990). Birth dates were assigned to natal hyenas by estimating their ages (± 7 days) when first observed above ground, based primarily on their pelage and size. We restricted our investigation to natal females that were at least 30 months of age, and immigrant males. Maternity assignments were based on genotyping and nursing associations, and paternity assignments were determined through genotyping alone, as described in Engh et al. (2002) and Van Horn et al. (2004). We assigned individuals social ranks based on the observed outcomes of dyadic agonistic interactions (Holekamp & Smale 1991; Smale et al. 1993). By convention, we assigned the highest-ranking individual in each intrasexual dominance hierarchy a rank of one.

**Observational and quantitative methods**

Throughout the study, behavioral observations were made twice daily, around dawn and dusk, when hyenas were socially most active (Kruuk 1972; Kolowski et al.

2007). Observations were made at dens, ungulate kills, and elsewhere within the clan's territory. At each observation session we recorded the identity of each hyena present, the time in minutes during which each hyena was observed, and all occurrences (Altmann 1974) of pasting, agonistic interactions, and greeting behaviors. Greeting is a common affiliative behavior among hyenas, wherein two individuals stand head-to-tail, lift their rear legs closest to the other, and sniff one another's anogenital region (Kruuk 1972; East et al. 1993).

For each session, we generated a male to female ratio by dividing the number of immigrant males present by the number of adult females present. Ratios were then categorized as high (> 1.3), medium (0.67 – 1.3), or low (< 0.67). Rates of pasting were calculated as the number of events per individual per hour observed (Woodmansee et al. 1991). Pasting rate data were not normally distributed and for many analyses could not be successfully transformed. Therefore, comparisons were made using nonparametric Friedman ANOVA, Mann-Whitney *U*, and Wilcoxon matched pairs tests (Statistica v6.1, 2002). As suggested by Mundry & Fischer (1998), we reported exact values (*T*) for Wilcoxon matched pairs tests with sample sizes less than 16, and asymptotic values (*Z*) for tests with sample sizes of 16 or greater. Holm's sequentially-rejective Bonferroni method was used to evaluate the statistical significance of multiple comparisons (Shaffer 1995). To assess the monotonic relationships between continuous variables, we utilized Spearman's rank correlation coefficients ($r_s$) (Statistica; Quinn & Keough 2002). For all analyses in which pasting rate was the dependent variable, individual hyenas were excluded if they were not observed for at least three hours in each pertinent independent

variable category. Descriptive statistics have been presented as mean ± standard error throughout.

Each pasting event was assigned a context based upon the social and behavioral events that occurred in the observation session during the preceding two minutes (Table 2.2). For each hyena, we calculated the proportion of its pasting events that occurred in each context. Proportion data were arcsin square root transformed and comparisons were made using paired and unpaired *t*-tests (Statistica). Hyenas were excluded from contextual analyses if they did not exhibit at least ten paste events.

**Data sets used in study (*A*, *B*, *C* and *D*)**

Given the broad scope of functional hypotheses addressed in this study, and the fact that it encompassed 12 years of behavioral observation data, multiple data sets were used to address the hypotheses (Table 2.1). The composition and value of each data set is explained below.

**Data set *A***

To obtain general descriptive information on pasting behavior, including the socio-ecological contexts in which it occurs among free-living adult hyenas, we studied its occurrence among 16 immigrant male and 12 natal female hyenas (Table 2.3). The immigrant males were studied from the time they were first seen interacting with members of the Talek clan until they either died or secondarily dispersed to another clan. Each of these males resided in the clan for at least two years, but died or secondarily dispersed before the end of the study. These criteria ensured that we obtained an accurate

Table 2.2. Socio-ecological contexts of pasting behavior considered in the current study. If more than one of these contexts preceded a paste event, we assigned the context that most immediately preceded the paste event.

| Context | abbr. | Description |
|---|---|---|
| spontaneous | spon | paste event was not preceded by evident stimuli |
| overmarking | om | paste placed on grass stalk scent marked previously by others in the same observation session |
| adjacent marking | am | paste event followed within two minutes of pasting by another, but paste was not placed on a grass stalk scent marked previously by others in the same observation session |
| agonism | agon | paste event followed a submissive and/or aggressive interaction with a social partner, or following a high-intensity conflict between clanmates in the same observation session |
| greeting | grt | paste event followed reciprocal leg-lifting and anogenital investigation with a social partner, or after offering or receiving a greeting invitation (i.e. leg-lifting) |
| other | oth | paste event preceded by rare stimuli, such as arriving in the observation session, participating in border patrols, inter-clan conflicts, lion – hyena conflicts, or allomarking of hyena cubs |
| unknown | unk | paste event was not preceded by two minutes of all-occurrence sampling |

Table 2.3. Time for which each hyena contributing to data set *A* was observed, as well as the number of paste events each hyena exhibited during this study.

| Name | Sex | Months Observed | Hours Observed | Pastes Observed | Alive, in clan, at study's end? (If yes, age in years) |
|---|---|---|---|---|---|
| BAIL | f | 70 | 204 | 46 | yes, 8.3 |
| BERN | f | 59 | 95 | 126 | yes, 7.4 |
| CR | f | 51 | 61 | 20 | yes, 6.8 |
| HOB | f | 65 | 97 | 42 | no |
| KIP | f | 48 | 131 | 65 | yes, 6.5 |
| MP | f | 40 | 77 | 18 | yes, 5.8 |
| PAR | f | 42 | 56 | 30 | yes, 6.8 |
| PT | f | 33 | 96 | 19 | no |
| SCY | f | 25 | 55 | 26 | no |
| SD | f | 34 | 87 | 50 | no |
| SEIN | f | 31 | 99 | 30 | yes, 5.1 |
| WR | f | 41 | 83 | 70 | yes, 5.9 |
| 9B | m | 32 | 37 | 6 | no |
| FA | m | 78 | 181 | 48 | no |
| FN | m | 104 | 218 | 103 | no |
| HOL | m | 55 | 118 | 34 | no |
| MARK | m | 31 | 28 | 2 | no |
| POS | m | 38 | 32 | 5 | no |
| QUAI | m | 58 | 118 | 67 | no |
| QUO | m | 28 | 42 | 19 | no |
| RCN | m | 76 | 121 | 23 | no |
| RS | m | 49 | 36 | 7 | no |
| SIM | m | 47 | 53 | 23 | no |
| STG | m | 56 | 36 | 11 | no |
| SY | m | 90 | 258 | 135 | no |
| TZ | m | 72 | 86 | 35 | no |
| VD | m | 26 | 24 | 23 | no |
| ZIP | m | 54 | 117 | 28 | no |

measure of each male's reproductive success within the Talek clan. We calculated each male's reproductive success as the number of cubs it sired divided by the total number of cubs born throughout its tenure. Cubs that had not been genotyped were excluded from these analyses. The 12 natal females were studied from the time they reached 30 months of age until either their death or the conclusion of the study. The initial selection criterion for these females was that they lived to be at least 4.5 years old. When multiple females from the same matriline met the initial criterion, we randomly selected one for study.

**Data set *B***

To best determine the effect of social status on pasting behavior we studied all the adult male (N = 15) and female (N = 20) hyenas present in the Talek clan from September, 1997 through June, 1999. During this time, both intrasexual dominance hierarchies remained stable, and there was minimal emigration or immigration by adult males. Pasting rates were calculated for each of the 35 adult hyenas during this period.

**Data set *C***

Although each female spotted hyena resides in a particular territory, she may occasionally be absent from her clan's territory for periods of days or weeks, presumably while on foraging or exploratory visits to other areas (Hofer & East 1993a,b,c; Holekamp et al. 1993). This data set allowed us to ascertain whether being separated from clanmates by absence from the clan's territory for extended periods of time affected scent marking activity. We studied all adult females that went unseen by observers in the Talek territory for periods of at least one month between 1989 and 1999. For each female studied, we

determined pasting rates during the two week periods preceding extended absences, and the two week periods following absences. Immigrant males were not studied because, once they had successfully immigrated into the clan, they were seldom absent for extended periods of time.

**Data set *D***

To evaluate the effect of reproductive state on female pasting behavior, we studied all females that successfully raised at least one offspring through weaning, between 1996 and 2000. Pasting rates were calculated for both pregnancy and lactation, defined as follows (Szykman et al. 2003):

Pregnancy (**p**): The gestation period in *Crocuta* lasts 110 days (Matthews 1939; Kruuk 1972). Therefore, females were considered pregnant for the 110 days preceding the births of their litters.

Lactation (**l1, l2, l3**): The length of the lactation period was determined from observations of nursing behavior and weaning conflicts (Szykman et al. 2003). As the length of the lactation period varies among female spotted hyenas (Holekamp et al. 1996; Holekamp & Smale 2000), we divided the overall lactation interval into three periods of equal length for each female (Szykman et al. 2003). Females often became pregnant while still nursing offspring from previous litters. When this occurred, females were categorized as pregnant, rather than lactating.

## RESULTS

Pasting rates among adult hyenas did not vary significantly with local prey abundance (data set *A*; Friedman ANOVA, $N_{male} = 16$, $\chi^2_{16,2} = 2.00$, $P = 0.368$; $N_{female} = 12$, $\chi^2_{12,2} = 3.50$, $P = 0.174$; Figure 2.1A). Additionally, pasting rates among adult males did not vary with rainfall (Friedman ANOVA, $\chi^2_{16,2} = 0.50$, $P = 0.779$; Figure 2.1B). Adult females, however, increased their scent marking activity during periods of high rainfall ($\chi^2_{12,2} = 13.17$, $P = 0.001$; Table 2.4). Although location did not significantly affect male pasting activity (Friedman ANOVA, $\chi^2_{16,2} = 2.63$, $P = 0.269$), adult females pasted more often at dens than at ungulate kills or elsewhere in their territory ($\chi^2_{12,2} = 12.67$, $P = 0.002$; Table 2.4, Figure 2.2). Given the elevated rates of pasting by females at dens, and the additional observation that lions were seldom seen at hyena dens during this study, we excluded observation sessions conducted at dens from an analysis evaluating the effect of lion presence on pasting behavior. The presence of lions in observation sessions inhibited pasting behavior in both males (Wilcoxon matched pairs, $N = 16$, $Z = 3.15$, $P = 0.002$), and females ($N = 12$, $T = 0.00$, $P < 0.001$; Figure 2.3).

Adult hyenas were observed pasting in a variety of socio-ecological contexts (Figure 2.4). Approximately one-third of the observed paste events were spontaneous. Overmarking and adjacent marking of conspecific scent marks were also relatively common, as was pasting immediately after agonistic interactions with clanmates. Within the context of agonism, neither males nor females pasted more frequently after interactions in which they were the socially dominant partner than after those in which they were subordinate (paired *t*-test, $N_{male} = 12$, $t = -1.88$, $P = 0.086$; $N_{female} = 12$, $t =$

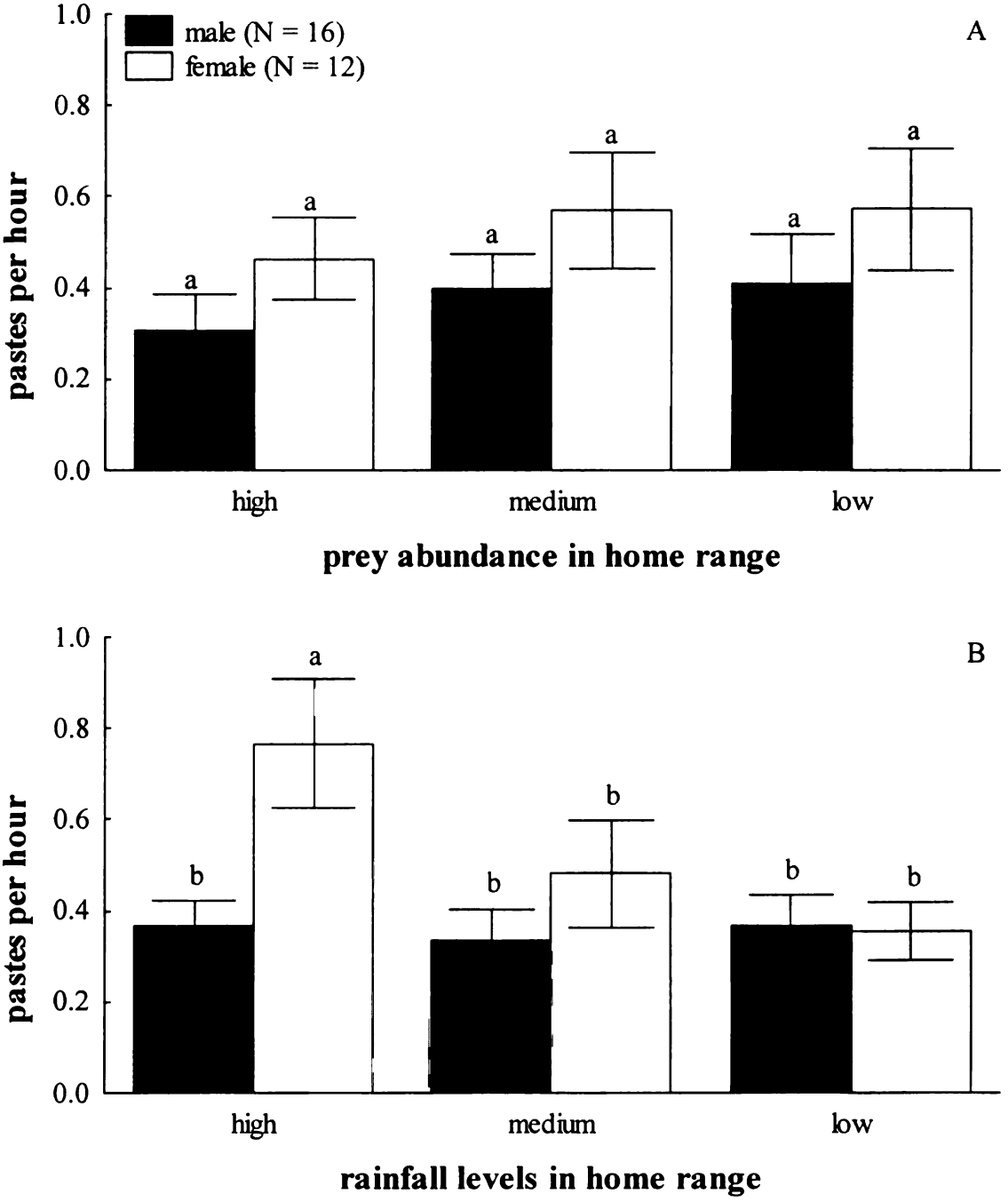



Figure 2.1. Mean (± SE) hourly pasting rates of adult spotted hyenas across periods of high, medium and low A) prey abundance, and B) rainfall within their home range. Letters above the error bars indicate statistically significant differences ($P < 0.05$).

Table 2.4. Results of Wilcoxon matched pairs tests comparing the hourly pasting rates of adult female hyenas across periods of high, medium and low rainfall, as well as at dens, ungulate kills and elsewhere (N = 12).

| Comparison | *T* | P – value |
|---|---|---|
| Rainfall levels | | |
| high vs. medium | 9.00 | 0.016 |
| high vs. low | 0.00 | 0.001 |
| medium vs. low | 28.00 | 0.424 |
| General location | | |
| den vs. kill | 1.00 | 0.001 |
| den vs. other | 8.00 | 0.012 |
| kill vs. other | 33.00 | 0.677 |

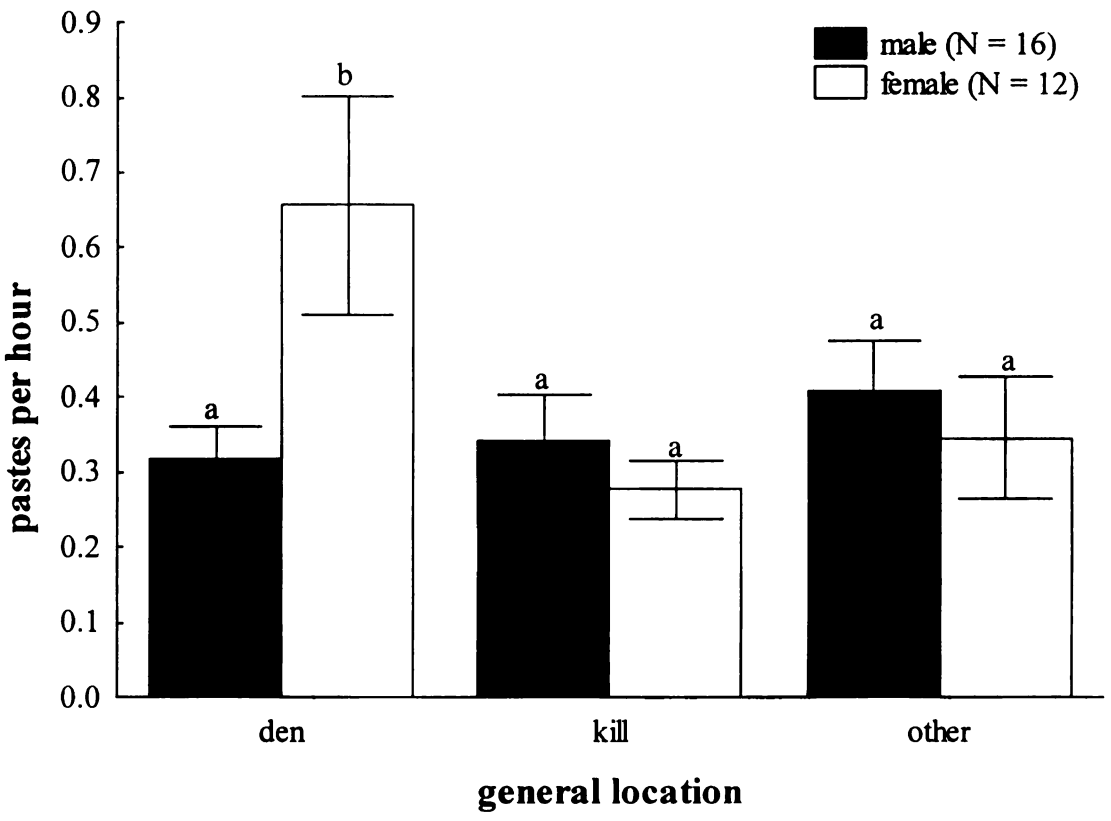



Figure 2.2. Mean (± SE) hourly pasting rates of adult male and female spotted hyenas at dens, ungulate kills, and elsewhere. Letters above the error bars indicate statistically significant differences ($P < 0.05$).

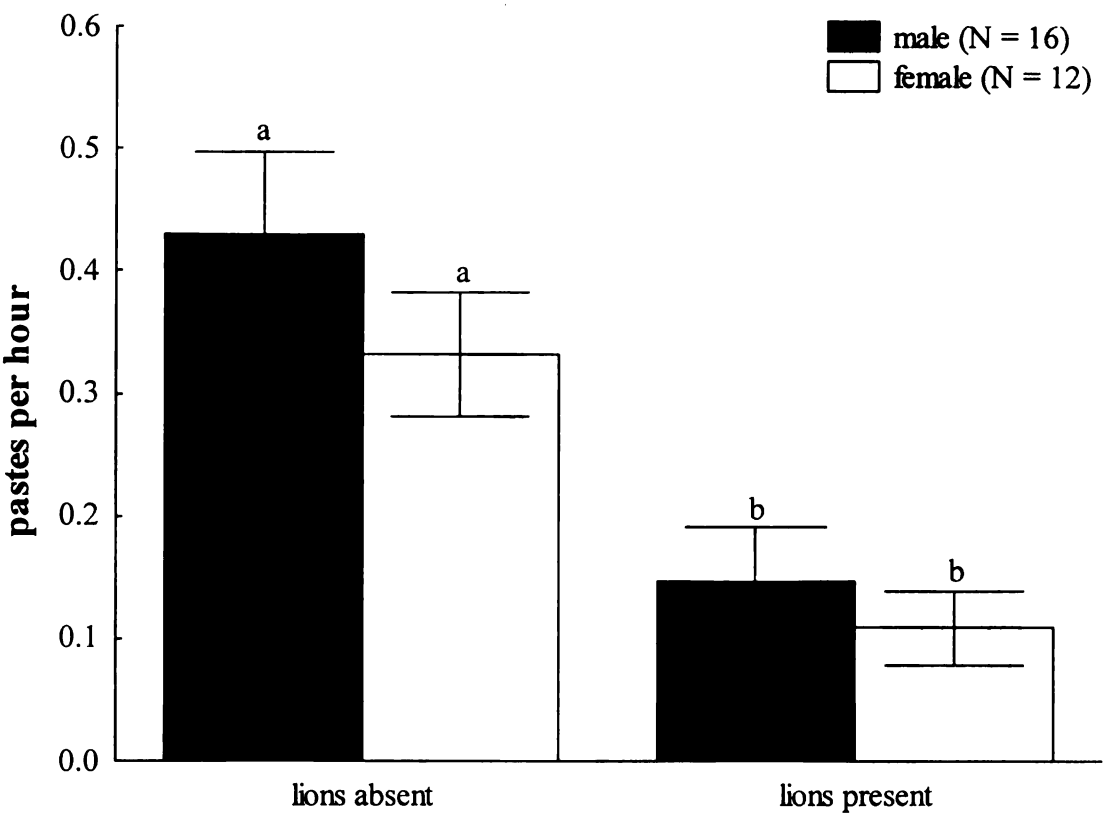



Figure 2.3. Mean (± SE) hourly pasting rates of adult male and female spotted hyenas during observation sessions at which lions were absent or present. Letters above the error bars indicate statistically significant differences ( $P < 0.05$).

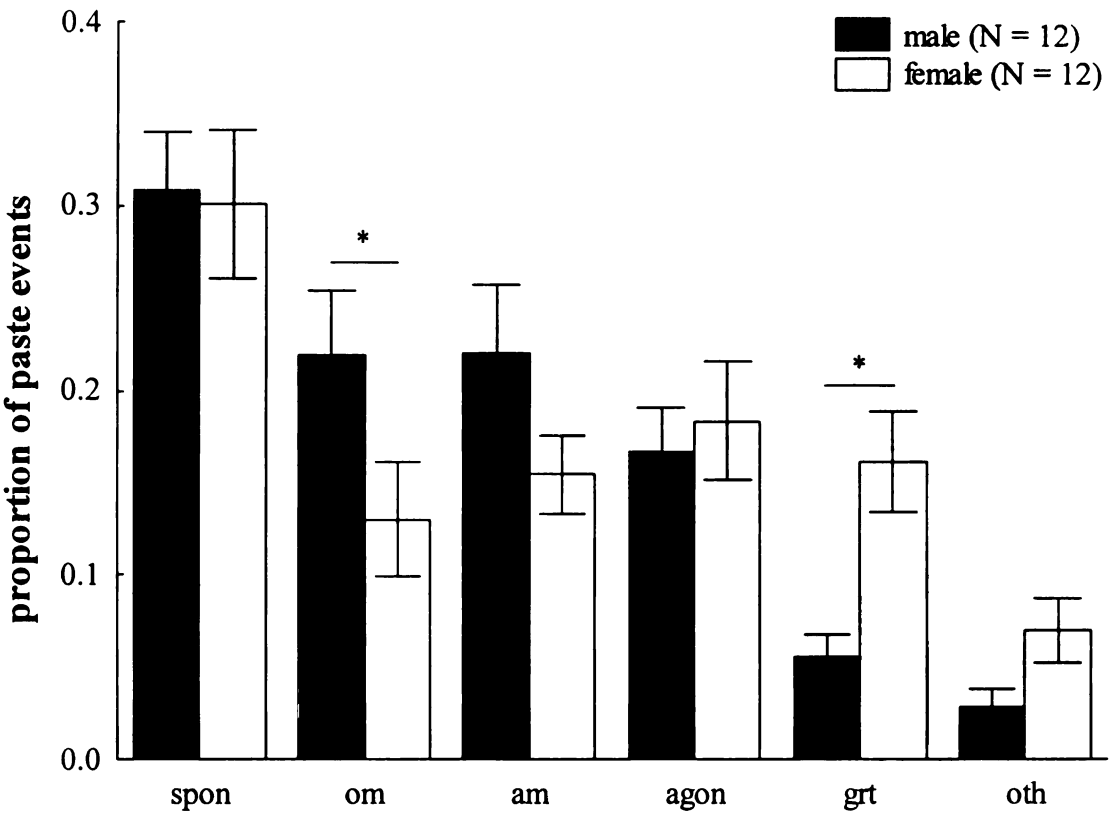



Figure 2.4. Socio-ecological contexts of pasting by adult male and female spotted hyenas. Data represent individuals observed to paste at least 10 times. The x-axis labels refer to spontaneous, overmarking, adjacent marking, agonism, greeting and other contexts, respectively. Approximately six percent of paste events were assigned an 'unknown' context. These events were excluded from all analyses. Asterisks indicate statistically significant differences ($P < 0.05$).

0.46, P = 0.652). Despite the substantial variability in the circumstances preceding pasting behavior, two sex differences in pasting context were evident. First, a greater proportion of female paste events followed participation in greeting behaviors (*t*-test, $t$ = 3.62, P = 0.002). Among both males and females, a majority of paste events in this context (75/109, 69%) occurred after individuals participated in full greetings (i.e. reciprocal leg-lifting and investigation of the anogenital region). The remainder of paste events in this context were equally represented by situations in which the pasting individual lifted its leg in invitation to another but the invitation was not reciprocated (18/109, 17%), and in which the pasting individual had received a greeting invitation that it did not reciprocate (16/109, 14%). The second evident sex difference in pasting context was that a greater proportion of male paste events were overmarks of conspecific scent marks ($t$ = -2.10, P = 0.047). Additionally, adult males and females overmarked the scents of different groups of conspecifics. Of the 113 observed instances of overmarking by adult males, 86 (76%) involved pasting over scent marks deposited by other adult males. Adult males did occasionally overmark scents deposited by juveniles (23, 20%), but they seldom overmarked adult females (4, 4%). Of the 54 observed instances of overmarking by adult females, 25 (46%) involved pasting over scents deposited by other adult females, and 29 (54%) involved scents deposited by juveniles.

**Testing predictions of $H_1$: Advertise dominance status**

Immigrant male hyenas pasted more frequently than did adult females (data set *B*; Mann-Whitney $U$, $N_{male}$ = 15, $N_{female}$ = 20, $U$ = 60.00, P = 0.002; Figure 2.5). Among males, we observed a strong positive correlation between social rank and pasting

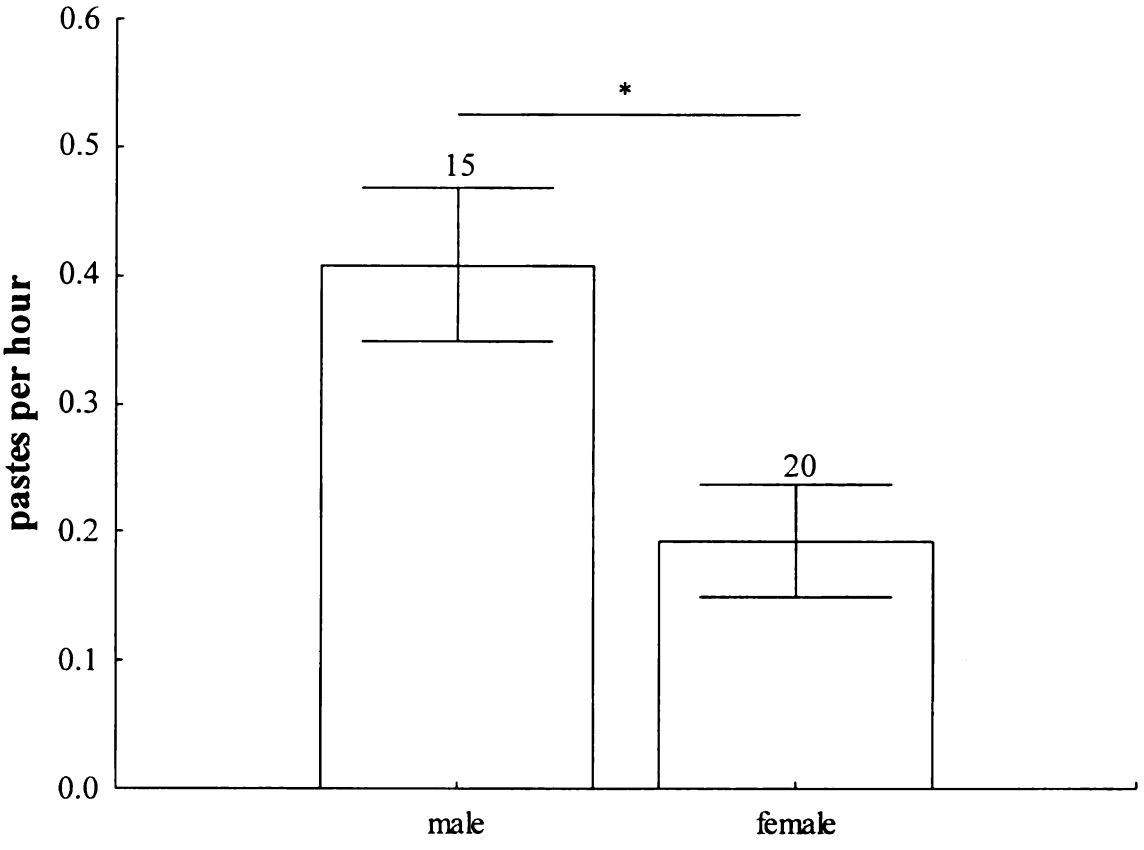



Figure 2.5. Mean (± SE) hourly pasting rates of adult male and female spotted hyenas residing concurrently in the same clan. The asterisk indicates a statistically significant difference ($P < 0.05$). Numbers above bars indicate individuals sampled.

rate (Spearman rank R, $r_s$ = -0.60, P = 0.018; Figure 2.6A), but no such correlation was evident among females ($r_s$ = -0.12, P = 0.616; Figure 2.6B). The proportion of paste events that were overmarks of conspecific scent marks was not correlated with intrasexual rank in either sex (male: $r_s$ = -0.33, P = 0.225; female: $r_s$ = -0.02, P = 0.946).

The ratio of adult males to adult females present in the immediate social environment had a marked effect on the frequency of scent marking by immigrant males. Males pasted most frequently during observation sessions in which their social partners were primarily other males (data set *A*; Friedman ANOVA, N = 16, $\chi^2_{16,2}$ = 8.00, P = 0.018; Table 2.5, Figure 2.7). For females, the effect of the ratio of males to females in sessions was not statistically significant (N = 7, $\chi^2_{7,2}$ = 5.43, P = 0.066), but this result appears to be a product of low sample size: 5 females were excluded from this analysis because they were too seldom present in high ratio sessions. Regardless, significant differences between males and females were evident. During observation sessions in which at least two-thirds of the hyenas present were male, males pasted at significantly higher rates than females (Mann-Whitney *U*, *U* = 17.00, P = 0.008). During sessions in which at least two-thirds of the individuals present were female, females pasted more frequently than males (*U* = 17.00, P = 0.008).

Interestingly, although the presence of adult male clanmates clearly elicited pasting behavior by immigrant males, the appearance in the Talek territory of new potential immigrant males did not appear to affect the frequency of pasting by established immigrant males. The hourly pasting rates of established immigrant males (data set *A*, but excluding data from first year of tenure), did not differ significantly between the two weeks after the appearance of new potential immigrant males in the territory and periods

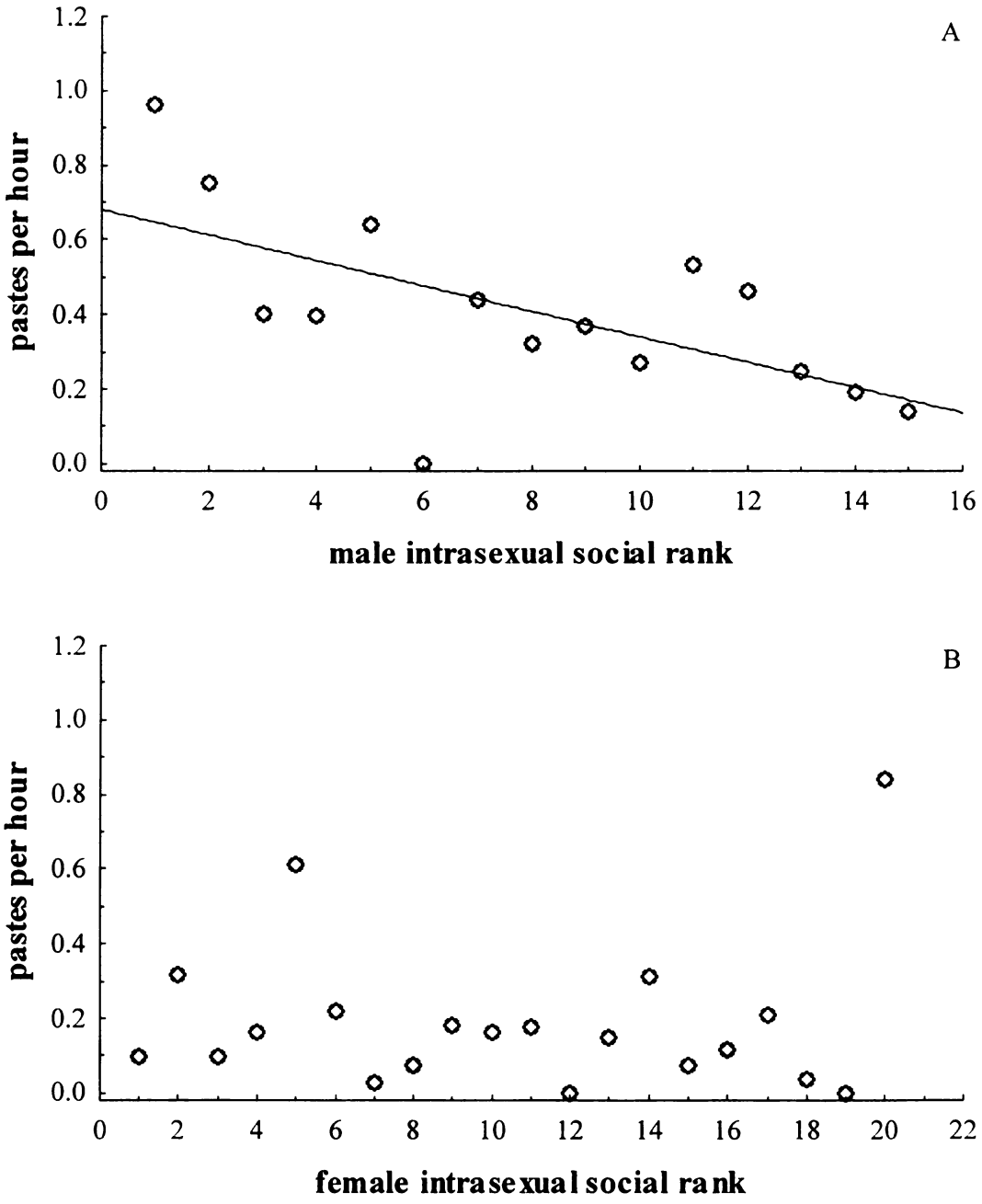



Figure 2.6. Effect of intrasexual social rank on rates of pasting behavior among adult A) male, and B) female spotted hyenas. By convention the highest-ranking member in each intrasexual hierarchy was assigned a rank of 1.

Table 2.5. Results of Wilcoxon matched pairs tests comparing hourly rates of pasting by adult male spotted hyenas in observation sessions characterized by high, medium and low ratios of adult males to adult females (N = 16).

| Comparison | *Z* | P – value |
|---|---|---|
| Ratio of males to females | | |
| high vs. medium | 2.28 | 0.023 |
| high vs. low | 2.79 | 0.005 |
| medium vs. low | 1.66 | 0.098 |

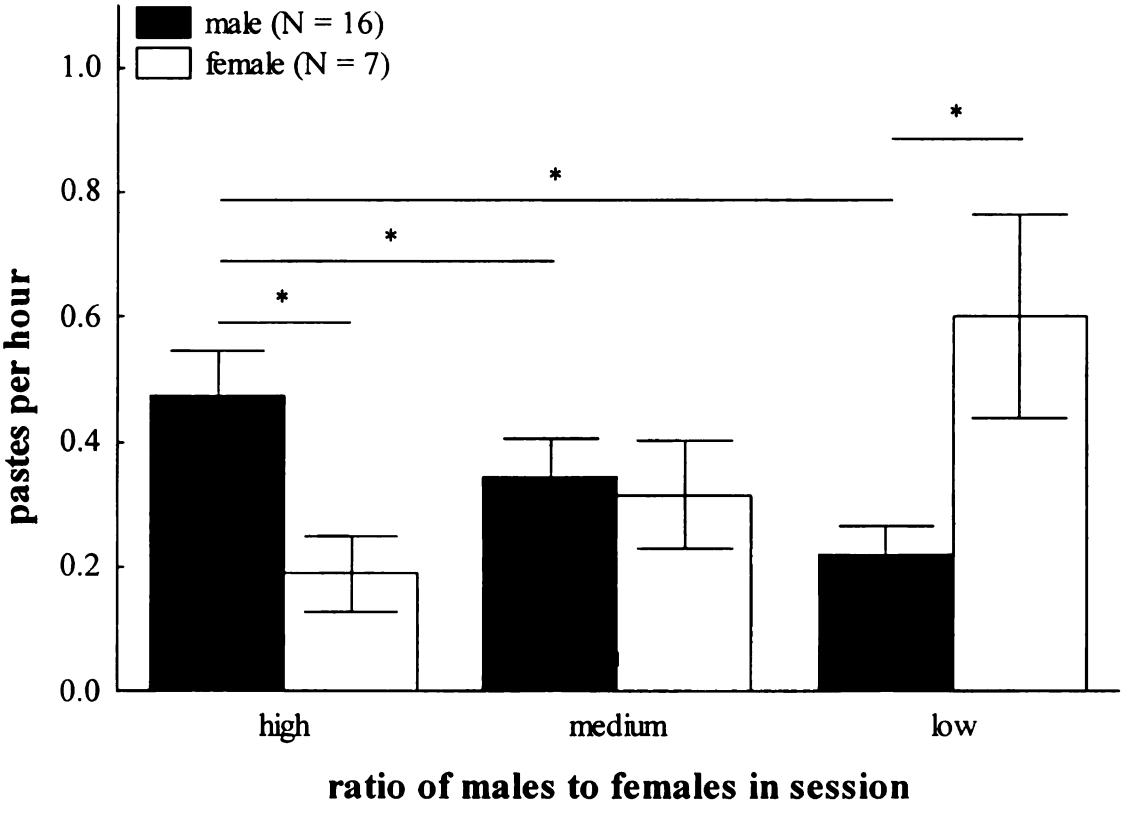



Figure 2.7. Effect of the sex composition among conspecifics in the immediate social environment on the frequency of pasting behavior by adult male and female spotted hyenas. Asterisks indicate statistically significant differences ($P < 0.05$).

in which no potential immigrants were observed (N = 15, Wilcoxon matched pairs, $T$ = 51, P = 0.639; new males: 0.42 ± 0.06, no new males: 0.43 ± 0.14).

**Testing predictions of $H_2$: Facilitate social cohesion**

From 1989 – 1999, nine resident adult females were absent from the Talek clan for periods of at least one month and were observed often enough to be included in the social cohesion analysis (7.6 ± 1.1 extended absences per female; 67.6 ± 15.3 days per absence). Eight of these females were very low-ranking socially, and one was mid-ranking. Among them, prolonged absence from the clan had a significant, positive effect on pasting rate (Figure 2.8). Females pasted approximately three times more often upon their return after an absence from the clan's territory of at least one month than they did prior to their departure (data set *C*; Wilcoxon matched pairs, N = 9, $T$ = 5.00, P = 0.039).

**Testing predictions of $H_3$: Advertise female sexual receptivity**

Although presumably physically capable (Matthews 1939; Holekamp et al. 1996), most of the females in data set *A* were not reproductively active at thirty months of age. From thirty months of age until onset of their first confirmed pregnancy, females pasted nearly three times more often than after they started breeding (Wilcoxon matched pairs, N = 10, $T$ = 1.00, P = 0.004; 0.92 ± 0.17 pastes / hr vs. 0.30 ± 0.05 pastes / hr). Among breeding females, pasting rates were not highest during late lactation, as predicted by this hypothesis; rather, females pasted considerably more often during pregnancy than during early, mid, or late lactation (data set *D*; Friedman ANOVA, N =

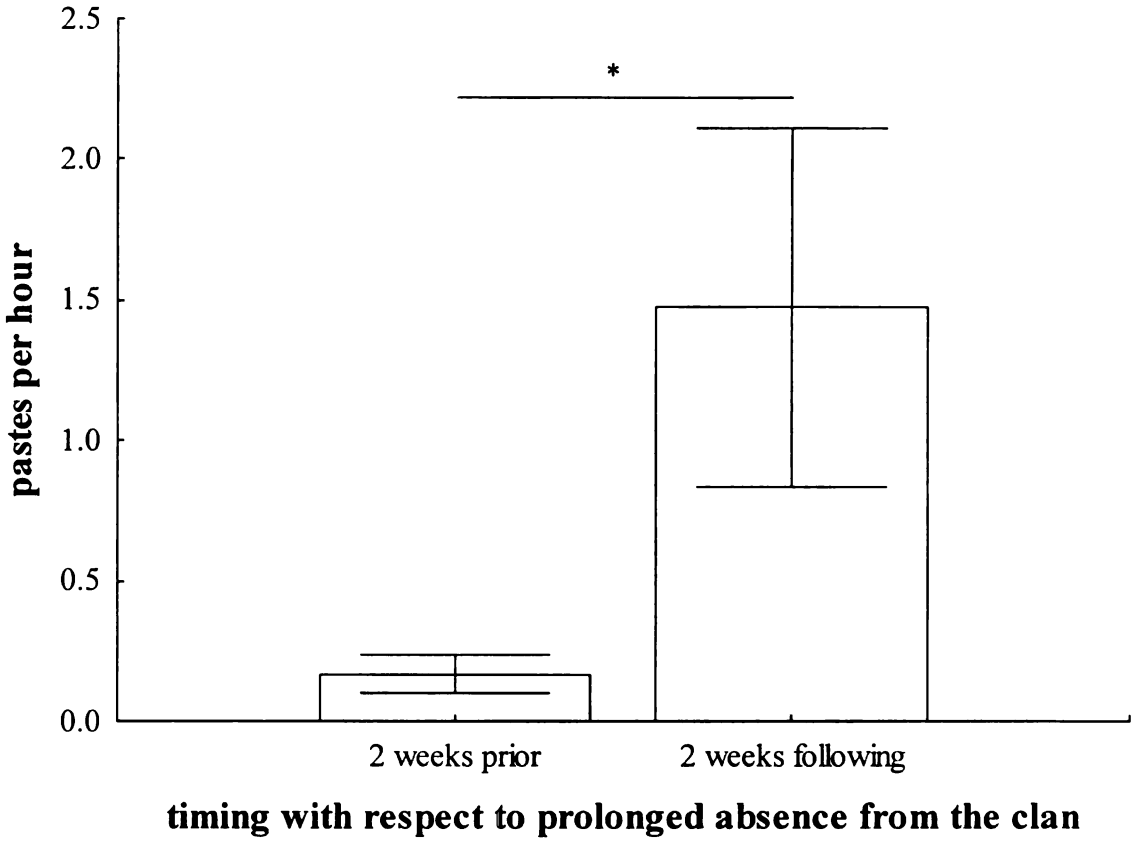



Figure 2.8. Mean (± SE) hourly pasting rates of adult female hyenas before and after absences from the Talek clan's territory lasting longer than one month (N = 9). The asterisk indicates a statistically significant difference ($P < 0.05$).

18, $\chi^2_{18,3} = 15.80$, P = 0.001; Table 2.6, Figure 2.9). During late lactation, females were seldom observed in sessions where no adult males were present, so we were unable to determine whether the presence of at least one male increased the likelihood of female scent marking behavior. However, females in late lactation did not significantly vary their pasting rates among sessions in which 1 – 2, 3 – 4, and 5 or more immigrant males were present (Friedman ANOVA, N = 10, $\chi^2_{10,2} = 3.07$, P = 0.215; Figure 2.10). Thus none of the predictions of this hypothesis were confirmed.

**Testing predictions of $H_4$: Advertise male availability for reproductive opportunities**

The rates at which immigrant males pasted during their first two years in their new clans was not significantly correlated with their relative reproductive success throughout their tenures (data set *A*; Spearman rank R, N = 16, $r_s = 0.02$, P = 0.956; Figure 2.11). Additionally, the rates at which immigrant males pasted in their third year of tenure and beyond were not significant predictors of relative reproductive success ($r_s$ = 0.20, P = 0.451). Lastly, the proportion of male pasting events that were overmarks of conspecific scent marks, in both their first two years in new clans and throughout the remainder of their tenures, did not appear to affect their reproductive success (first two years: $r_s = -0.21$, P = 0.444; remainder of tenure: N = 14, $r_s = 0.10$, P = 0.742). Thus, as with $H_3$, none of the predictions of this hypothesis were confirmed by our data.

Table 2.6. Results of Wilcoxon matched pairs tests comparing the hourly pasting rates of adult female hyenas across various reproductive states (N = 18).

| Comparison | *Z* | P – value |
|---|---|---|
| Reproductive state | | |
| pregnancy vs. early lactation | 3.42 | < 0.001 |
| pregnancy vs. mid lactation | 3.59 | < 0.001 |
| pregnancy vs. late lactation | 2.24 | 0.025 |
| early vs. mid lactation | 0.97 | 0.332 |
| early vs. late lactation | 0.37 | 0.711 |
| mid vs. late lactation | 1.14 | 0.256 |

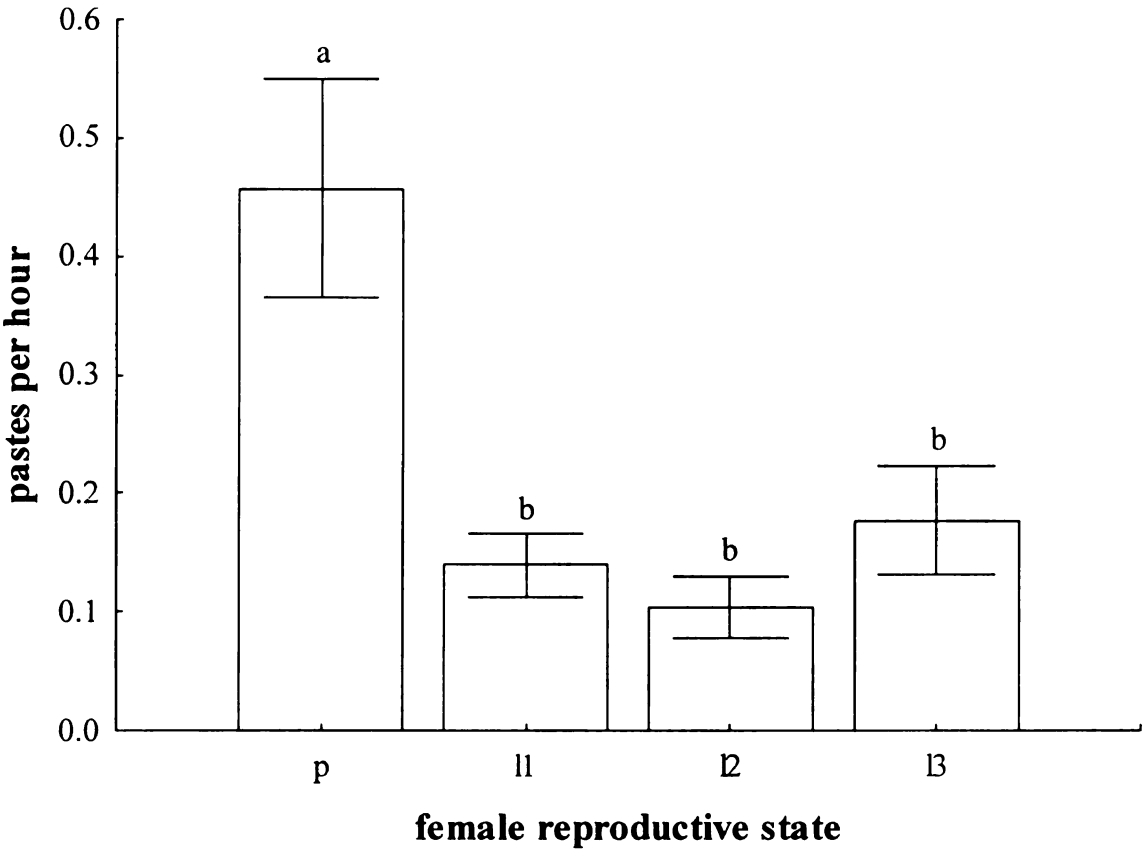



Figure 2.9. Mean (± SE) hourly pasting rates of female spotted hyenas during pregnancy, early (l1), mid (l2), and late (l3) lactation (N = 18). Letters above the error bars indicate statistically significant differences ($P < 0.05$).

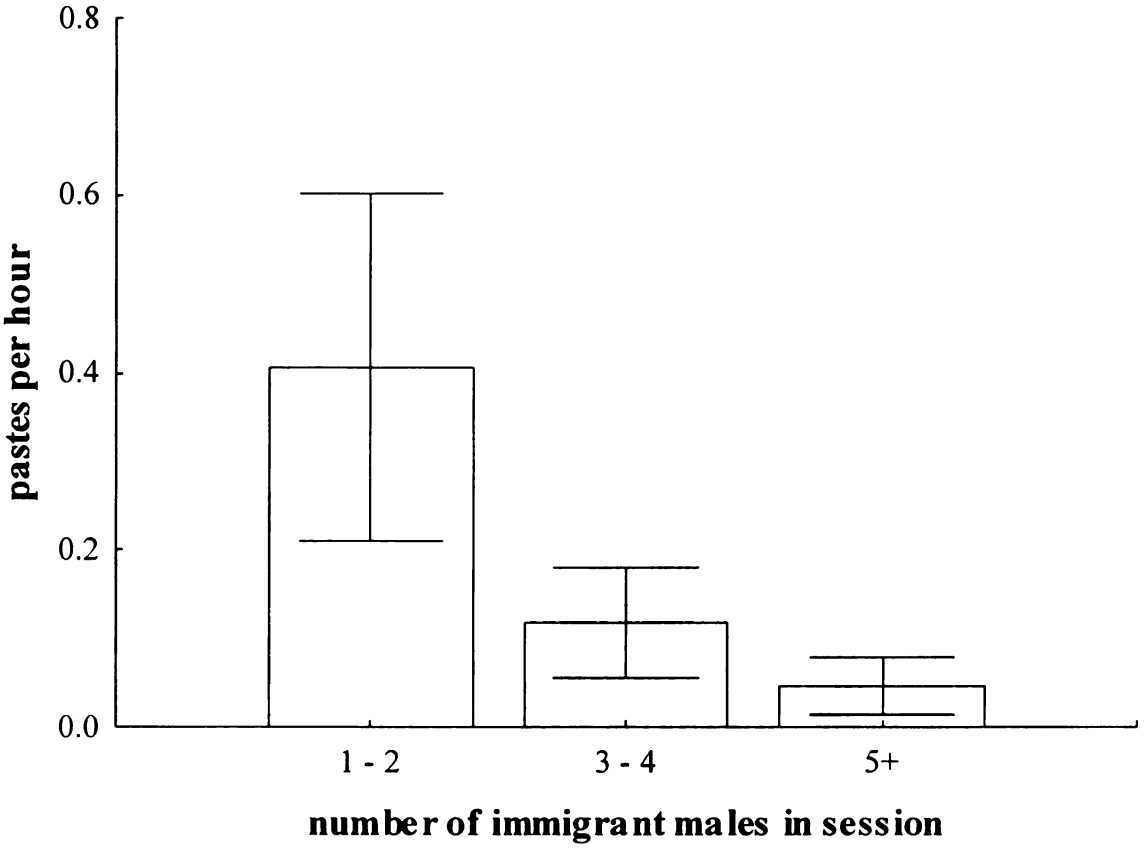



Figure 2.10. Mean (± SE) hourly pasting rates of female spotted hyenas during late lactation during observation sessions in which 1 – 2, 3 – 4, or more than 4 immigrant males were present (N = 10). Differences were not statistically significant.

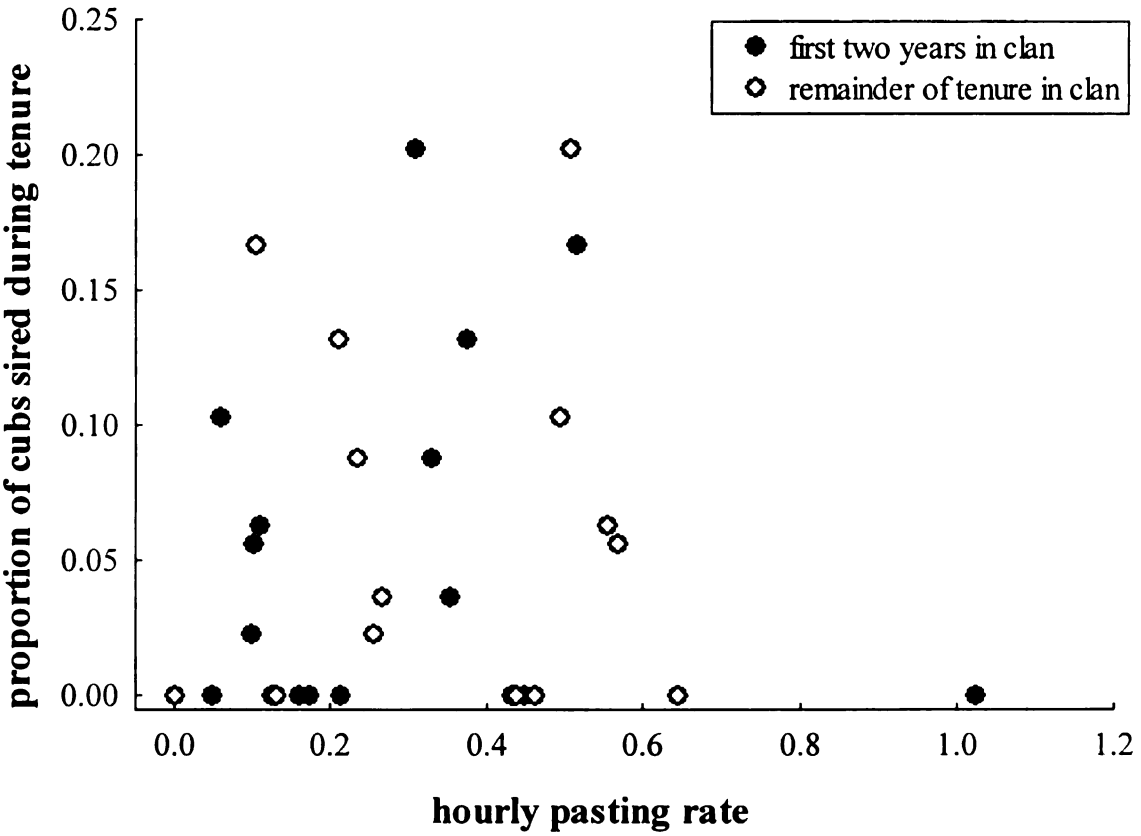



Figure 2.11. Relative reproductive success of immigrant male spotted hyenas in relation to pasting rate during the first two years of residency and during the remainder of each male's tenure in their new clans (N = 16).

## DISCUSSION

Scent marking figures prominently in territorial maintenance and defense for many mammalian species (Gosling 1982; Gorman & Trowbridge 1989). However, placing exclusive emphasis on the territorial function of scent marking in gregarious mammals potentially overshadows important intragroup functions of scent marking, such as social integration or reproductive advertisement (Eisenberg & Kleiman 1972; Hofer et al. 2001). It has been suggested that pasting behavior in the spotted hyena functions exclusively in territorial maintenance and defense (Gorman & Mills 1984). Here we have demonstrated that, in addition to functioning in territorial defense, pasting has multiple intragroup functions and that at least some of these functions are sexually dimorphic. Our analyses support the hypothesis that adult immigrant males, but not adult females, advertise their dominance status via pasting. They further suggest that females utilize pasting to facilitate social cohesion within clans. Unfortunately, our data were insufficient to permit us to test this hypothesis among adult males. Lastly, our data fail to support hypotheses suggesting that pasting advertises female sexual receptivity or that it serves as a form of male sexual advertisement.

### Assessment of $H_1$: Advertising dominance status

Scent marking advertises dominance status to competitors and potential mates in many mammals (Ralls 1971; Gosling & Roberts 2001; Hurst & Beynon 2004). However, noting that immigrant males pasted more frequently than other age/sex classes of spotted hyena, and that immigrant males are the lowest-ranking members of hyena societies, previous researchers concluded that pasting does not function to advertise dominance

status among adult spotted hyenas (Mills & Gorman 1987). In this study, we have confirmed that, as is typical among mammals (Johnson 1973), adult male spotted hyenas scent mark more often than their female peers. However, we have further demonstrated a positive correlation between intrasexual social rank and the frequency of scent marking among males. We did not find a correlation between rank and scent marking frequency among females, however. Although a definitive explanation for this dimorphism will require further investigation, a preliminary supposition is that using scent marking to advertise dominance status is less important for maintaining hierarchies formed among philopatric individuals than it is for maintaining those formed among immigrants, as the latter need to routinely accommodate the addition of strangers. Interestingly, the same sexually dimorphic patterns in scent marking frequency have been observed in the ring-tailed lemur (*Lemur catta*), another gregarious mammal with male-biased dispersal and female-dominated societies (Kappeler 1990; Sussman 1992; Drea & Scordato 2008).

A common method used to advertise dominance status among male mammals is to engage in competitive scent marking or 'scent wars' (Gosling & Roberts 2001; Hurst & Beynon 2004), in which an individual ensures that the preponderance of recently deposited scent marks in an area are its own rather than those of its competitors (Rich & Hurst 1998, 1999). A high quality individual can ensure that its marks predominate by physically excluding competitors from the area, discouraging competitors who are in the area from engaging in scent marking themselves, and/or by effectively countermarking scent marks deposited in the area by competitors (Rich & Hurst 1998, 1999). Although male spotted hyenas attempting to immigrate into new clans can be viciously attacked by males who have already successfully immigrated into those clans, this is uncommon

(Smale et al. 1997; East & Hofer 2001). Furthermore, hyena clans typically contain multiple breeding males that exhibit very low rates of aggression amongst themselves (Kruuk 1972; Frank 1986b; Mills 1990; Smale et al. 1997; East & Hofer 2001). Additionally, we have not observed low-ranking immigrant males being attacked by higher-ranking males subsequent to scent marking by the former. Given that socially dominant male hyenas mark at the highest rates, that male pasting rates are highest in the vicinity of male competitors, and that one-half of all pasting events exhibited by males in this study were countermarks (overmarks + adjacent marks) of clanmates' scent marks, it appears that males are advertising their dominance status through the sheer volume of their marking and potentially through their effectiveness in countermarking male competitors as well. In the current study, the vast majority of overmarking by immigrant males appeared to be directed toward the scent marks of other adult males. However, before it can be definitively asserted that immigrant males are targeting the scent marks of other males for overmarking, the opportunities available for males to overmark scents laid down by different age/sex classes of hyena need to be controlled.

**Assessment of $H_2$: Facilitate social cohesion**

The social cohesion hypothesis for scent marking suggests that it facilitates the benefits, and modulates the costs, of group-living by reaffirming individual presence and membership within groups, thereby strengthening the affiliative ties that exist among group members (modified from Willis & Brigham 2004; Sharpe 2005; McCowan et al. 2008). Persistent reaffirmation of presence is believed to be crucial in maintaining familiarity and social tolerance among group members in gregarious species (East et al.

1993; Hurst 2005; McCowan et al. 2008). For example, when subordinate male house mice (*Mus musculus*) are experimentally prevented from contributing their odors to the substrates shared by members of their groups, they are attacked more quickly and harshly than control males (Hurst et al. 1993). Similarly, naked mole-rats (*Heterocephalus glaber*) react aggressively to colony members that have been removed from their burrow systems for only twelve hours, suggesting that odor familiarity needs to be continually reinforced within mole-rat societies (O'Riain & Jarvis 1997).

Maintaining social cohesion may be particularly challenging for large groups of animals, particularly those living in fission-fusion societies wherein long-term associations must be maintained despite frequent, and sometimes lengthy, physical separation of group members (Willis & Brigham 2004; Lehmann et al. 2007). Spotted hyena clans are large fission-fusion societies in which members travel, rest and forage in subgroups that change in composition multiple times each day (Smith et al. 2008). In fact, it is exceedingly rare for all members of medium and large size hyena clans to be concurrently present in the same area (Holekamp et al. 1997; Smith et al. 2008).

As predicted by East et al. (1993), in the current study we found that females that had engaged in extended absences from their clans pasted far more often upon returning than they did prior to departing. This suggests that pasting may facilitate re-assimilation into clans and enhance social cohesion. Among spotted hyenas, greeting ceremonies are particularly common during subgroup reunions, and extrusion of the anal pouch during spotted hyena greeting ceremonies is most common among clanmates who meet least frequently (East et al. 1993; Hofer et al. 2001). One-fifth of female pasting events in the

current study occurred just after participation in greeting, further suggesting that pasting facilitates social cohesion in hyenas clans, at least among adult females.

Two additional findings of this study lend support to the social cohesion hypothesis for pasting among adult female hyenas. First, female pasting rates were highest in the vicinity of dens (see also Hofer et al. 2001). Communal dens are the social centers of hyena societies and are frequently attended by most clan members (Boydston et al. 2006; White 2007). They are therefore ideal places at which to reaffirm one's presence within the group and strengthen affiliative ties. Interestingly, in the current study, the observed positive effect of rainfall on female scent marking behavior remains when we considered only observation sessions at dens (unpublished data). Given that rainfall degrades scent marks (Alberts 1992), the increased pasting activity by females at dens during periods of high rainfall further suggests the importance to females of effectively communicating their presence in the area to clanmates.

The second additional finding that lends support to the social cohesion hypothesis is that females in the current study pasted particularly often during pregnancy. This is important to the current argument for two reasons. First, pregnant females are more apt than females in other reproductive conditions to take extended absences from their clans' home ranges (Holekamp et al. 1993). Second, given that communal dens are the social centers of hyena clans, they pose significant risks of aggression to mid- and low-ranking females and, of greatest consequence to the argument, their offspring (Boydston et al. 2006; White 2007). Investment in self-advertisement and reaffirmation of group membership prior to giving birth may be of particular value. In the future, it should be determined whether, controlled for social rank, investment in scent marking while

pregnant reaps rewards in terms of reduced rates of aggression directed towards oneself and one's offspring at communal dens.

**Assessment of $H_3$: Advertise female sexual receptivity**

Chemical advertisement of estrus status has been widely observed among female mammals (Johnson 1973; Johansson & Jones 2007). Advertising estrus may be particularly important for females in species with female-dominated societies, as advertising sexual receptivity may encourage the approach of apprehensive males, thereby increasing the likelihood of fertilization (Petrulis & Johnston 1997; Palagi et al. 2004). Chemical cues to estrus do appear to exist in the spotted hyena (Szykman et al. 2001); however, our analyses suggest they may not be available in paste. In the current study, females did not paste most often during late lactation, the reproductive period during which female hyenas are most likely to be in estrus. Instead, female hyenas pasted most often when they were pregnant. Additionally, during late lactation, female pasting activity did not vary with the degree of male presence in observation sessions, further suggesting that they were not signaling sexual receptivity to immigrant males.

Our results are consistent with the idea that scent marking is likely an inefficient means through which to communicate information about estrus in highly social mammals, given that prospective mates are oftentimes in the immediate vicinity of females (Wolff et al. 2002; Ferkin et al. 2004). Although male hyenas likely cannot perfectly monitor the reproductive status of all females in their clans (Engh et al. 2002), males have frequent contact with most female clanmates (Szykman et al. 2001), and males are often observed sniffing females directly and sniffing areas in which females

have recently urinated or lain down (Frank 1986b). Although we currently have a relatively poor understanding of the female reproductive cycle in *Crocuta*, estrus in female hyenas appears to last for 24 – 48 hrs, and seems to reoccur every two weeks, barring fertilization (East et al. 2003). As paste readily persists in the environment for a month or more (Gorman & Mills 1984), pasting would not be an ideal means by which to chemically advertise estrus in this species. Urine is a much more likely candidate, given that mammalian urine contains numerous steroid metabolites (Hauser et al. 2008), and that the biological significance of estrus-specific urinary products has been demonstrated in a wide variety of mammals (e.g. Michael 1975; Swaisgood et al. 2002; Slade et al. 2003; Rajanarayanan & Archunan 2004; Achiraman & Archunan 2006).

**Assessment of $H_4$: Advertise male availability for reproductive opportunities**

Among immigrant male spotted hyenas, reproductive success is dependent to a large degree upon a male's length of tenure in his current clan (Engh et al. 2002; East et al. 2003). If female spotted hyenas are considering the length of time males have been members of their clans when making mate choice decisions (Honer et al. 2007), then males would seemingly benefit from advertising their presence in clans early and often, as doing so would potentially increase females' familiarity with their scents and increase the likelihood that they will be considered for mating opportunities in the future (Mills & Gorman 1987). In the current study, we have, to our knowledge, provided the first evaluation of the potential effect of scent marking frequency on the long-term reproductive success of male mammals in a wild population. The frequency at which male hyenas pasted early, as well as late, in their tenures was unrelated to their relative

reproductive success. Moreover, we found that the degree to which males invested in overmarking conspecific paste also did not affect their reproductive success.

Only persistently motivated, high-quality males can saturate their environments with scent marks (Fisher et al. 2003). Therefore, females that base mate choice decisions on their familiarity with individual male odors, the frequencies at which they encounter the odors of individual males, or how consistently they observe individual males placing scent marks atop those of other males, presumably select highly competitive, genetically sound mates (Johnston et al. 1997; Fisher et al. 2003; Ferkin et al. 2005; Cheetham et al. 2008). However, even females in species that do not use these criteria when selecting mates can obtain valuable information about the suitability of potential mates from male scent marks (Mech et al. 2003). Information about male quality can be obtained directly from a single scent mark if the marks of dominant, high-quality males include compounds, or levels of compounds, unique to their condition (e.g. Hayes et al. 2001). Furthermore, male scent marks potentially provide females with opportunities to assess the genetic compatibility of potential mates (reviewed by Thom et al. 2008). Given that genetic compatibility is potentially driving mate choice decisions in the spotted hyena (East et al. 2003), the role pasting by immigrant males plays in allowing females to assess their genetic compatibility with prospective mates needs to be determined.

CHAPTER THREE

# DISCRIMINATION OF CONSPECIFIC SCENT BY FREE-LIVING SPOTTED HYENAS (*CROCUTA CROCUTA*)

## INTRODUCTION

An effective communication system is a critical component of the behavioral repertoire of nearly every animal species. Among mammals, a particularly common form of communication is scent marking, which is the deliberate deposition of urine, feces and/or the products of exocrine scent glands in the environment for the purpose of signaling conspecifics (Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973). Mammalian scent marking can serve to facilitate the defense of resources, maintain dominance and social relationships, attract mates, or promote reproductive synchrony (Ralls 1971; Eisenberg & Kleiman 1972; Johnson 1973; Gosling & Roberts 2001; Rich & Hurst 1998). However, the functions of scent marking vary widely, not only among mammalian species, but also among different age, sex, reproductive and social classes within a single species (e.g. Begg et al. 2003; Miller et al. 2003; Swaisgood et al. 2004; Hurst 2005; Lewis 2006; Jordan 2007). Additionally, it has become increasingly clear that we need to develop a more comprehensive understanding of the functions of scent marking in highly gregarious species (Scordato & Drea 2007; Muller & Manser 2008), especially given that social complexity potentially drives signaling complexity (e.g. Freeberg 2006). Therefore, the purpose of the current study was to further our understanding of the functions of scent marking in a highly gregarious large carnivore, the spotted hyena, *Crocuta crocuta*.

The functions of animal signals are best ascertained by identifying the broad and specific socioecological contexts in which the signals are emitted, and by determining the types of information the signals transmit (Yahr 1983; Drea et al. 2002). In a previous study, we described the contexts in which spotted hyenas exhibit scent marking behavior (Theis et al. *submitted*). In the current study, we extended our previous findings by conducting scent discrimination experiments with free-living spotted hyenas to determine the information available in hyena scent marks.

Spotted hyenas live in complex social groups, called clans, that typically contain 40 – 80 individuals (Kruuk 1972; Trinkel et al. 2007). Hyena clans are fission-fusion societies, wherein members are seldom all present in the same place at the same time; rather members fission into subgroups that change in composition and size several times each day (Holekamp et al. 1997; Smith et al. 2008). Hyena clans contain multiple breeding males and multiple overlapping generations of females. In contrast to most mammalian social systems, hyena societies are female-dominated, and social status within a clan's linear dominance hierarchy is not determined by size or fighting ability (Frank 1986a,b). Instead, natal hyenas 'inherit' the social ranks of their mothers (Frank 1986b; Holekamp & Smale 1991, 1993; Smale et al. 1993; Engh et al. 2000), and adult immigrant males, all of which are subordinate to natal individuals, queue for dominance status with other immigrant males (Smale et al. 1997; East & Hofer 2001).

To mediate the complex social relationships between and within clans, spotted hyenas utilize a rich array of vocal, visual and chemical signaling behaviors (Kruuk 1972; Mills 1990). A common and conspicuous form of chemical signaling among spotted hyenas is 'pasting' behavior (Matthews 1939). When pasting, hyenas extrude their anal

scent pouches and drag them over grass stalks. In doing so, hyenas typically deposit anal gland secretions near the tops of the grass stalks (Matthews 1939; Kruuk 1972; Mills 1990). These secretions potentially remain as viable signals for more than a month (Gorman & Mills 1984).

Previous investigations of pasting among spotted hyenas have shown that it figures prominently in territorial maintenance. In high-density populations, spotted hyenas demarcate the boundaries of their territories with pastings, often engaging in border patrols wherein several hyenas collectively engage in high rates of scent marking along their clan's territorial boundaries (Kruuk 1972; Henschel & Skinner 1991; Boydston et al. 2001). In low-density hyena populations, members of clans are likely unable to effectively replenish scent marks along their territorial borders and, consequently, instead engage in hinterland marking, concentrating their pasting activity beside key resources within their territories rather than along territorial boundaries (Mills & Gorman 1987). Additionally, it has been shown that captive spotted hyenas investigate paste from donors with which they are unfamiliar more often than they do paste from familiar donors (Drea et al. 2002), and it has been reported anecdotally that *Crocuta* in natural populations react strongly to scent marks from non-clanmates found in their territories. However, no one has yet systematically demonstrated that free-living spotted hyenas discriminate between the scents of clanmates and others. Therefore, the first objective of the current study was to determine whether hyenas discriminate between paste samples obtained from clanmate and non-clanmate donors, and to affirm that they spend more time investigating scents from non-clanmates.

The second objective of the current study was to discern whether the ability of free-living hyenas to discriminate between the scents of clanmates and others is based on the degree of familiarity between respondents and donors, or is instead based on chemical badges of group membership available in paste. Again, captive hyenas discriminated between paste samples from donors with which they were familiar and samples from donors with which they were unfamiliar (Drea et al. 2002). However, previous investigations have also revealed that the chemical compositions of paste samples from members of the same clans are more similar than the chemical compositions of samples from members of different clans (Hofer et al. 2001; Burgener et al. 2008). Thus, spotted hyena paste potentially contains information about the donor's group membership. If discrimination between paste samples from clanmates and non-clanmates is based on familiarity, then hyenas should additionally discriminate between samples obtained from members of neighboring clans and those obtained from members of distant clans (Leger 1993). If discrimination is based on the presence of group-specific odors in paste, then hyenas should discriminate between paste samples obtained from members of two distant clans with which the respondents are presumably equally unfamiliar. Note that these mechanistic hypotheses are not mutually exclusive.

In addition to facilitating territorial maintenance, several intragroup functions have been proposed for spotted hyena pasting as well. Specifically, it has been suggested that pasting serves to advertise reproductive condition (Mills 1990; Drea et al. 2002), to advertise dominance status (Woodmansee et al. 1991; Theis et al. *submitted*), and to facilitate social cohesion within clans (East et al. 1993; Theis et al. *submitted*). Given that these hypotheses primarily involve signaling potential mates or same-sex competitors,

they imply that paste should convey information on donor sex. Drea et al. (2002) showed this to be the case, as captive adult spotted hyenas discriminated between paste samples from male and female donors. The third objective of the current study was to verify this finding with free-living hyenas. A reproductive function for pasting implies that, at least among females, paste contains information about reproductive state. This is not necessarily so for male hyenas, as they are in reproduction condition year round once they reach adulthood (Matthews 1939). In many mammals, the scent marks of females provide males with information about their reproductive condition and receptivity (Johnson 1973; Johansson & Jones 2007). The fourth objective of this study was therefore to ascertain whether such information is available in the paste produced by females, by determining whether hyenas discriminate between paste samples from pregnant and lactating female donors. A final objective of this study was to determine, when possible, whether these discriminative abilities or tendencies are age- or sex-dependent.

## METHODS

### Study site and subjects

This study was conducted in the Masai Mara National Reserve, Kenya. The Reserve consists of rolling grassland, scattered brushland, and narrow stretches of riparian forest, and supports sizable concentrations of resident and migratory ungulates (Frank 1986a; Holekamp et al. 1999; Cooper et al. 1999). From June – December, 2004, we conducted scent discrimination experiments on members of three distinct hyena clans in the Reserve: West Talek, East Talek, and Mara River clans (Figure 3.1). Prior to 2000,

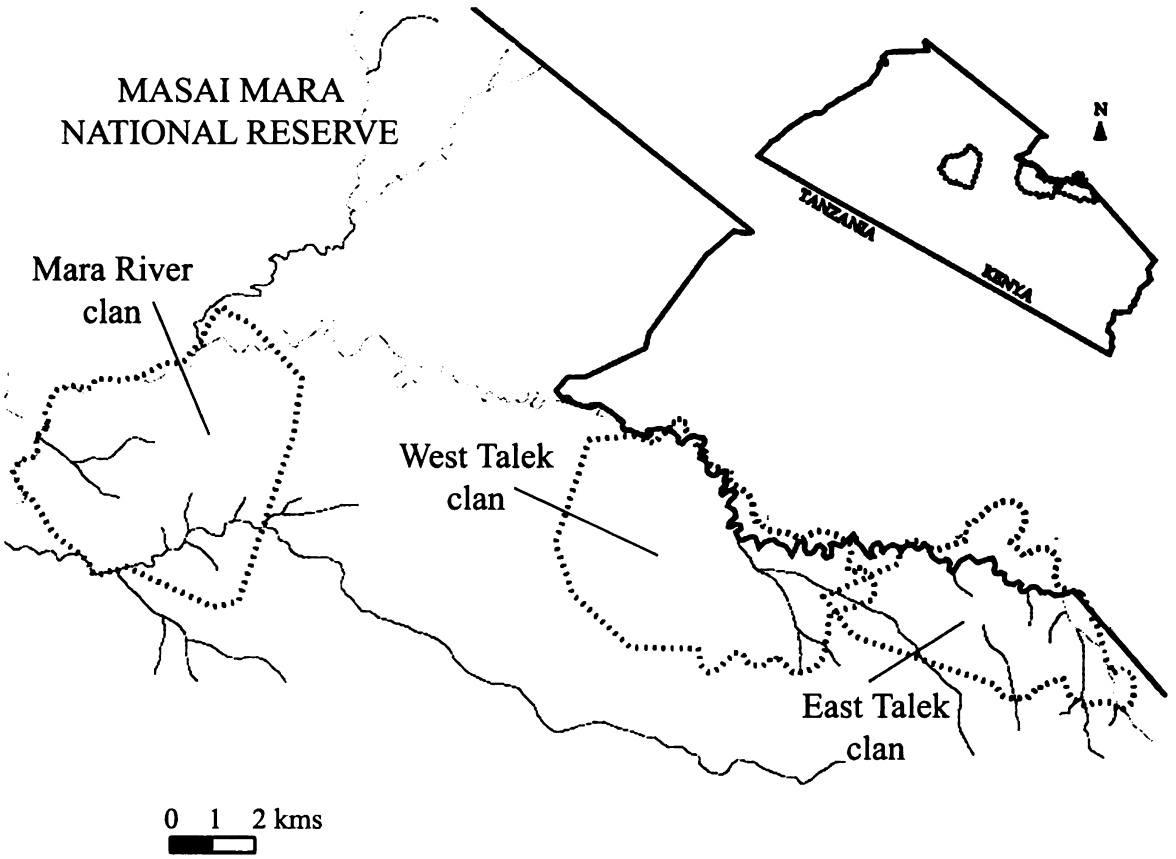



Figure 3.1. Map illustrating the locations in the Reserve of the three clans used in the current study. The territory sizes of the Mara River, West Talek, and East Talek clans were 31 $km^2$, 28 $km^2$, and 19 $km^2$, respectively (Kolowski et al. 2007). During the study period there were 30 hyenas present in the Mara River clan, 51 in the West Talek clan, and 32 in the East Talek clan. This map was adapted, with permission, from Kolowski et al. (2007).

there was a single clan in the Talek region of the Reserve. By 2002, however, the hyenas comprising the Talek clan had completely fissioned into two separate, yet contiguous clans, which we now refer to as the West and East Talek clans (J.E. Smith, Michigan State University, unpublished data). The Mara River clan resides approximately 10 km to the west of the West Talek clan.

The hyenas in these three clans had been previously habituated to the presence of our research vehicles. Clan members were individually identified by their unique spot patterns and other conspicuous physical characteristics. Sex was determined from the dimorphic glans morphology of the erect phallus, as described in Frank et al. (1990). Birth dates were assigned to natal hyenas by estimating their ages (± 7 days) when first observed above ground at dens, based primarily on their pelage and size. In this study, hyenas less than 12 months of age were considered to be cubs, and those between 13 and 30 months old were categorized as subadults. Together, cubs and subadults were called "juveniles." All natal females greater than 30 months of age were considered to be adults, as were all immigrant males.

**Sample collection and storage**

Paste samples were obtained directly from the anal scent pouches of anaesthetized adult spotted hyenas sampled throughout much of the Reserve between 1994 and 2004. Scent donors were immobilized with Telazol (W.A. Butler; 6.5 mg/kg), delivered from a $CO_2$-powered darting rifle (Telinject), and paste samples were scraped directly from their anal pouches with a sterilized scalpel handle. Upon collection, paste samples were placed in sterile cryogenic vials (Corning). The vials were placed in liquid nitrogen and

remained continuously frozen until being used in experiments. The age of samples in this study refers to the time between date of sample collection from an immobilized hyena and date of use in a scent discrimination experiment. We utilized 63 samples from 60 different hyenas. We made aliquots of the frozen samples (25/63 samples) that were to be used multiple times during the study.

Since the chemical composition of scent marks can change over time (Gorman 1976; Buesching et al. 2002; Cavaggioni et al. 2006), it is ideal for frozen samples to be thawed shortly before their use in scent discrimination experiments. Given the logistics of conducting scent discrimination experiments here with free-living large carnivores, there were occasional instances in which experiments were postponed at the last moment, after aliquots had already been thawed. Delays occurred as a result of rainfall, car trouble, human and wildlife interference, and hyenas having moved their den sites without our knowledge. When delays occurred, we used the aliquots in experiments as soon as conditions permitted; usually the next evening. Overall, most aliquots were used in experiments within one hour of being removed from the liquid nitrogen tank (54/88, 61%), and the vast majority (82/88, 93%) were used by the next day. Six aliquots were used in experiments four days after being thawed. Although this was not ideal, hyena paste is a very long-lasting signal that remains potent in the environment for a month or more (Gorman & Mills 1984; Apps et al. 1989). Most importantly, in every trial during this study, the paired paste aliquots had been thawed for exactly the same amount of time. On average, aliquots were set out 2.2 ± 0.3 hrs before hyenas arrived at the test sites.

**Location and setup of experiments**

The scent discrimination experiments were conducted primarily at communal den sites within the territories of the three study clans (39/44 trials, 89%). Communal dens are the social centers of hyena societies and are attended by most clan members each evening (Boydston et al. 2006; White 2007). They are therefore ideal locations for maximizing participation in experiments. There was a period of approximately one month during the study in which the Mara River clan did not have an active communal den. To conduct experiments with this clan during that time, we located its members before dawn and, based on their direction of travel, anticipated where they would rest during the day. We then drove ahead of the traveling hyenas and set up the experiments beside the anticipated resting sites (5/44 trials, 11%).

In this study, we conducted both three- and two-choice scent discrimination experiments. In the three-choice discrimination experiments (e.g. Fisher et al. 2003), we presented subjects with three new 70-cm segments of flexible, plastic-covered electrical wire (no. 7 AWG) positioned vertically in the ground with approximately 55 cm of each wire remaining exposed. The wires were placed one meter apart in a straight line, and were positioned perpendicular to the current wind direction, and as upwind as possible, of the den or resting site. Each wire was first thoroughly wiped with 70% isopropyl alcohol. We then used clean wooden applicators to apply paste samples (~0.05 g), 5 cm from the tops of two wires. The height was chosen because *Crocuta* typically place paste marks 40 – 50 cm high on grass stalks (Kruuk 1972). The top of the third wire was rubbed with a clean wooden applicator and served as a control. The positioning of samples among the three wires was randomized.

Two-choice discrimination experiments (e.g. Smith et al. 1997; Swaisgood et al. 2004), were conducted toward the end of this study for two reasons. First, as noted above, there was a period of time when the Mara River clan did not have an active den site and we had to rely on the behavioral cues of subjects to predict appropriate locations for testing sites. Conserving time during setup of the experiment was a prime concern during this period. Second, by this time it had become clear that the scent samples themselves, not the experimental apparatus, were primarily driving olfactory investigation of the wires, so including control wires in experiments was no longer informative (see Results).

There was only a single instance of a sample being used twice within the same experiment. The second time it was used, it was paired with a different sample than the one with which it had been paired originally, and the second trial was also conducted with a different clan. There was also a single instance of a sample being presented twice in testing of the same clan. The second presentation was for a different experiment and occurred more than two months after the sample had first been presented. Only two of the eight participants in the latter trial had participated in the first trial. Excluding the responses of these two individuals in the latter trial would not have changed the statistical significance of any result in that experiment.

**Descriptions of discrimination experiments**

**Clanmate vs. non-clanmate:** In the first experiment we tested whether spotted hyenas preferentially investigate the scents of non-clanmates over those of clanmates. We conducted 11 trials in which we presented participants with paste samples from a clanmate, a non-clanmate, and a control simultaneously. In seven of the trials the paste

donors were female, and in four they were male. We defined a "neighboring" clan as one with a territory abutting that of the tested clan, and a "distant" clan as one separated from the territory of the tested clan by at least one other territory. The non-clanmate paste donor was a member of a neighboring clan in four trials, and was from a distant clan in the other seven trials. Eight trials were conducted with West Talek, two with East Talek, and one with the Mara River clan.

**Neighboring vs. distant clan:** In the second experiment we sought to determine whether hyenas discriminate between scents from neighbors and donors from distant clans. We conducted 10 trials in which we presented participants with paste samples from a neighbor, a member of a distant clan, and a control simultaneously. All donors from neighboring clans were confirmed members of their respective clans within the previous year. In six of the trials the paste donors were female, and in four they were male. Seven trials were conducted with the East Talek clan and three were conducted with the West Talek clan.

**Distant vs. distant clan:** In the third experiment we attempted to ascertain whether hyenas discriminate between the scents of two distant clans. We assumed that the experimental participants had had no prior contact with scent donors from either distant clan. We conducted six trials with the Mara River clan in which we presented participants with paste from a member of the West Talek clan, paste from a same-sex member of the East Talek clan, and a control simultaneously. In four of the trials the paste donors were female, and in two they were male.

**Male vs. female:** In the fourth experiment we determined whether free-living hyenas discriminate between the scents of males and females. We conducted 10 trials in which

we simultaneously presented participants with paste samples from an adult male and female that were both members of the same distant clan. Each of the female donors were lactating when sampled. Five trials were conducted with the West Talek clan and five were conducted with the Mara River clan.

**Pregnant vs. late lactating:** In the final experiment we sought to find out whether hyenas discriminate between paste samples from pregnant females and those sampled during late lactation. We conducted 7 trials in which we simultaneously presented participants with samples from two female clanmates, one pregnant, the other in late lactation. Pregnancy was defined as the 110 days preceding parturition (Matthews 1939; Kruuk 1972). Since the mean weaning age in *Crocuta* is 14 months (Holekamp et al. 1996), late lactation was defined as the period between seven months post-parturition and weaning. Five trials were conducted with the Mara River clan and two were conducted with the East Talek clan.

**Data collection**

As most of the discrimination trials were conducted at night (37/44, 84%), we dictated observations into a digital voice recorder and transcribed our observations the following morning. When transcribing, we determined the lengths of sniffing (nose within 10 cm of stimulus) bouts by participants by timing our vocalized observations with a stopwatch. All nighttime observations were made through night vision goggles (AN/PVS-7 Generation 3), supplemented with an infrared light source (SONY DCR-TRV530 digital handycam with a SONY HVL-IRH2 video infrared light).

We recorded the presence and identity of every hyena that approached within 5 m downwind of the experimental stimuli. This represented the vast majority of animals in the general vicinity of the experiments; only a few hyenas remained at the peripheries of the sites and departed before their identities could be confirmed. Given that the fur of newborn hyena cubs is typically uniformly black, they are generally difficult to individually identify using night vision goggles. Therefore, unless they bore some feature that facilitated our readily identifying them as individuals, newborn cubs were not included in analyses.

When a hyena approached the stimuli, we recorded its direction of approach with respect to the wind. Although exceptionally rare, if a hyena approached the experimental apparatus from upwind, its responses were excluded from analyses for that experiment, because hyenas approaching from upwind may be responding primarily to visual, rather than chemical, stimuli. For each hyena, we recorded the total time spent sniffing each of the stimuli, as well as any incidences of overmarking the stimuli (pasting on an experimental wire). Occasionally, two hyenas concurrently investigated the stimuli. When this occurred, to avoid potential bias due to social facilitation, we only recorded the response of the first hyena to approach the stimuli. If the other hyena returned to the stimuli at some point later in the trial, we then recorded data from it.

**Conclusion of experiments**

Trials concluded following 1) prolonged periods of inactivity at sites after substantial participation (1 ± 0.1 hrs; 17/44, 39%), 2) participants pulled an experimental wire from the ground (16/44, 36%), 3) substantial rainfall (9/44, 20 %), and, 4) on two

occasions, equipment failure (5%). Note that, experimental trials did not conclude as soon as any overmarking of the stimuli occurred (see also Palphramand & White 2007). Although the application of additional scents on experimental stimuli can potentially influence subsequent responses by other participants (Bel et al. 1999), given the logistic challenges of the current study, we found it unfeasible to conclude trials as soon as overmarking occurred.

As most trials were conducted at communal den sites, the earliest participants in them were usually cubs, who tended to emerge from den holes shortly before their mothers and older siblings arrived. After investigating the stimuli, cubs commonly overmarked them. Therefore, had we ended trials after the first incidence of overmarking, we could not have obtained response data from subadults or adults. Through pilot trials, we had learned that young hyenas even attempted to overmark samples on inflexible wooden dowels. The wooden dowels inevitably snapped and, not only did we then lose exceptionally rare and valuable scent samples, but the splintered dowels also posed an injury risk to the hyenas. Therefore, we chose instead to present the samples on electrical wires, as they re-assume their original vertical position after being overmarked and can subsequently be investigated further by other participants. Also, it should be noted that most spotted hyena cubs and subadults do not consistently produce paste in their anal glands (Theis et al. 2008). Therefore, it is unlikely that they were actually depositing paste when overmarking experimental stimuli. Only once during the entire study did an adult hyena overmark a stimulus. Nevertheless, for each discriminative ability that hyenas exhibited in this study, we attempted to verify that overmarking had not been a

factor by determining whether participants exhibited the discriminative ability prior to any overmarking of stimuli having occurred.

In some trials, members of each age/sex class were not able to participate because an early participant had pulled a wire from the ground, there was exceptionally poor turnout at the test site, or rainfall occurred early during the trial. In cases with poor turnout or rainfall, if no overmarking of the stimuli had yet occurred, we retrieved the wires, secured the top of each with a Ziploc bag, stored the wires in a cool dry place overnight, and used them again, at the same site, the following evening (4/44, 9%). In instances where rainfall occurred, we quickly retrieved the wires and, in each case, paste samples remained clearly visible on them. If a wire had been pulled from the ground or overmarking of stimuli had occurred, and we had additional aliquots of the samples, new wires and new samples were utilized the following night instead (5/44, 11%). Whenever trials were replicated in these ways, we only used the data from individual hyenas that were obtained during their first evening interacting with the stimuli. Whenever it could be done without unduly disturbing the hyenas, we removed the experimental wires from the test site immediately following the conclusion of a trial. When this was not possible, the wires were removed very early the next morning.

**Quantitative analyses**

An inherent challenge with stationary field experiments on free-living animal subjects is that researchers cannot control the arrivals and departures of individual participants. Consequently, the lengths of time participants were present varies substantially across trials. Additionally, the numbers of trials at which individual

participants were present within each experiment varies. As a result, the total time individuals spent sniffing samples varied considerably. Therefore, in an effort to standardize responses and to avoid pseudoreplication, we calculated the proportion of time each individual hyena spent sniffing each stimulus type, out of the total time spent sniffing all stimuli combined, across all trials within an experiment. Our sampling unit in all experiments was the individual hyena.

Proportions could not be consistently normalized, so we employed nonparametric statistical analyses (Statistica, v6.1, 2002) . We used Friedman ANOVA tests to assess treatment effects for 3-choice scent bioassays. If treatment effects were statistically significant, we evaluated whether hyenas discriminated between non-control samples by using Wilcoxon matched pairs tests (*T*-statistic; Mundry & Fischer 1998). Wilcoxon matched pairs tests were also used to evaluate the outcomes of 2-choice bioassays. All statistical tests were two-tailed except for Wilcoxon matched pairs tests in which we assessed the differential investigation of samples from clanmates and non-clanmates because a directional response had been predicted. When comparing two independent sampling groups, we utilized Mann-Whitney *U* tests. Descriptive statistics have been presented as mean ± standard error throughout.

## RESULTS

Cubs and subadults frequently participated in the scent discrimination experiments (Figure 3.2). Participation by adults was less common, with adult females participating at moderate levels and immigrant males doing so only rarely. The lack of participation by immigrant males seemed due primarily to their low levels of attendance

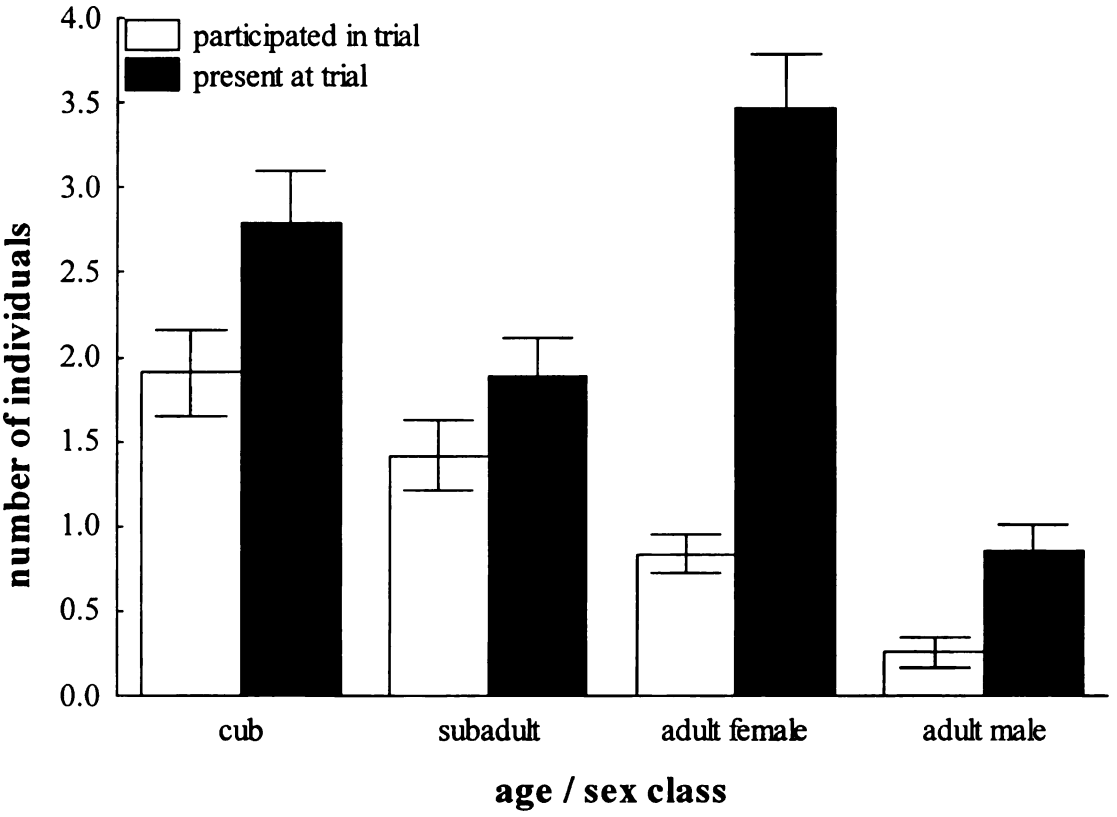



Figure 3.2. The presence and participation of different age / sex classes of spotted hyena across all 44 scent discrimination trials conducted in this study.

at den sites, and their remaining at the peripheries of den areas when they were in attendance (see also East & Hofer 1991, 1995; White 2007). Given that immigrant males seldom participated in experiments, we excluded them from all statistical analyses, although we have included descriptions of their responses when available.

**Age of paste samples**

In this study, the age of samples used in experiments ranged from one month to ten years (3.3 ± 0.2 yrs). Sample age did not affect the degree to which samples were investigated by respondents. Analyses of samples from each class of scent donor revealed that the time elapsed since sample collection did not affect the amount of time during which samples were sniffed by participating hyenas (Table 3.1).

**Clanmate vs. non-clanmate**

Significant treatment effects were evident for cubs (Friedman ANOVA, N = 14, $\chi^2_{14,2} = 7.00$, P = 0.03), and subadults (N = 13, $\chi^2_{13,2} = 7.88$, P = 0.019), but not adult females (N = 9, $\chi^2_{9,2} = 0.81$, P = 0.666; Figure 3.3). Only a single immigrant male participated in this experiment, and he exclusively investigated paste samples from non-clanmates. The significant treatment effects among juveniles were due primarily to their lack of investigating control wires, as neither cubs (Wilcoxon matched pairs, $T = 32.00$, P = 0.109 (one-sided)), nor subadults ($T = 35.00$, P = 0.377), investigated paste samples from non-clanmates more than samples from clanmates. However, considering only trials in which paste samples from female donors were used, both cubs (N = 11, $T = 10.50$, P = 0.023 (one-sided)) and subadults (N = 9, $T = 8.00$, P = 0.049) investigated paste samples

Table 3.1. Results of Spearman's rank tests assessing the monotonic relationship between the age of paste samples and the amount of time they were investigated. Investigation times were calculated by averaging the responses of all participating hyenas toward each paste sample for a given trial. Therefore, sample sizes indicate the number of paste samples per donor class used in each experiment. Within each experiment, samples from the two scent donor classes were analyzed separately.

| Experiment | Donor class | N | $r_s$ | P-value |
|---|---|---|---|---|
| clanmate vs. non-clanmate | | | | |
| | clanmate | 11 | -0.22 | 0.520 |
| | non-clanmate | 11 | -0.04 | 0.916 |
| neighboring clan vs. distant clan | | | | |
| | neighboring clan | 10 | -0.30 | 0.404 |
| | distant clan | 10 | 0.19 | 0.604 |
| distant clan vs. distant clan | | | | |
| | West Talek | 6 | -0.03 | 0.958 |
| | East Talek | 6 | -0.37 | 0.469 |
| male vs. female | | | | |
| | male | 10 | -0.25 | 0.489 |
| | female | 10 | -0.20 | 0.580 |
| pregnant vs. late lactating | | | | |
| | pregnant | 7 | 0.29 | 0.535 |
| | late lactating | 7 | 0.54 | 0.216 |

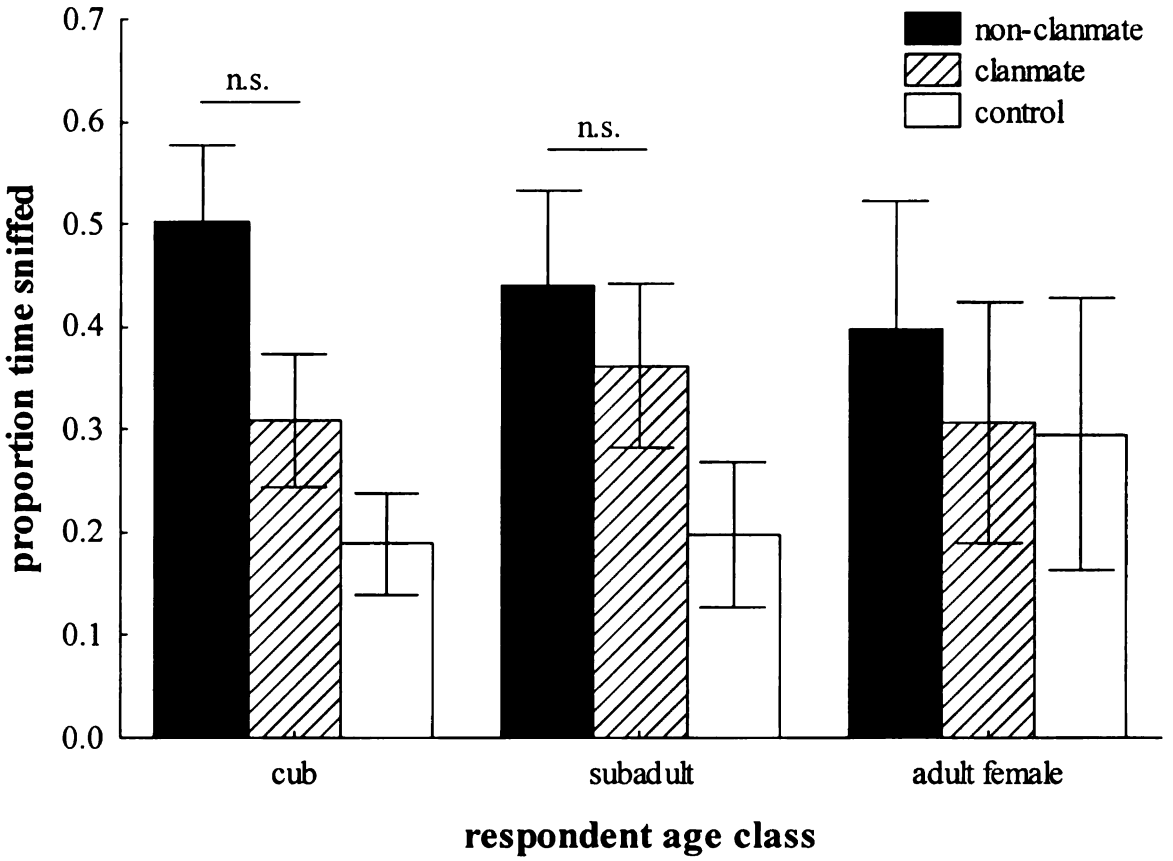



Figure 3.3. The relative amount of time cub, subadult and adult female spotted hyenas spent sniffing paste samples from non-clanmates, clanmates, and control wires. "n.s." indicates a statistically non-significant difference between time spent sniffing clanmate and non-clanmate odors, following a significant overall treatment effect for that age class ($P < 0.05$). Sample sizes for cubs, subadults and adult females are 14, 13 and 9, respectively.

from non-clanmates more than those from clanmates (Figure 3.4). Among juveniles collectively, the effect of donor clan membership on relative investigation time was evident before any overmarking of experimental stimuli occurred (N = 8, $T = 0.00$, P = 0.004 (one-sided)).

Ten juveniles (8 cubs, 2 subadults) overmarked an experimental stimulus at least once. They did not differentially overmark paste samples from non-clanmates, clanmates, or the control wires (Friedman ANOVA, $\chi^2_{10,2} = 0.94$, P = 0.625). There was also no significant effect of treatment on overmarking activity when considering only trials using female scent donors ($\chi^2_{7,2} = 1.00$, P = 0.607).

**Neighboring clan vs. distant clan**

Significant treatment effects were again evident for cubs (Friedman ANOVA, N = 12, $\chi^2_{12,2} = 6.68$, P = 0.035), and subadults (N = 10, $\chi^2_{10,2} = 7.19$, P = 0.027), but not adult females (N = 5, $\chi^2_{5,2} = 3.60$, P = 0.165; Figure 3.5). The significant treatment effect observed with cubs was again due to minimal investigation of the control wires, as they did not differentially investigate paste samples obtained from neighboring and distant clan members (Wilcoxon matched pairs, $T = 18.00$, P = 0.110). This lack of discrimination remained when considering only trials using scent from female donors (N = 12, $T = 33.00$, P = 0.677). Subadults also did not differentially investigate samples from neighboring and distant clan members (all trials: $T = 21.00$, P = 0.557; female donor trials only: N = 5, $T = 7.00$, P = 1.000). Three immigrant males participated in this experiment, and each sniffed paste samples from neighboring hyenas more than samples from members of distant clans. Six juveniles (3 cubs, 3 subadults) overmarked an experimental

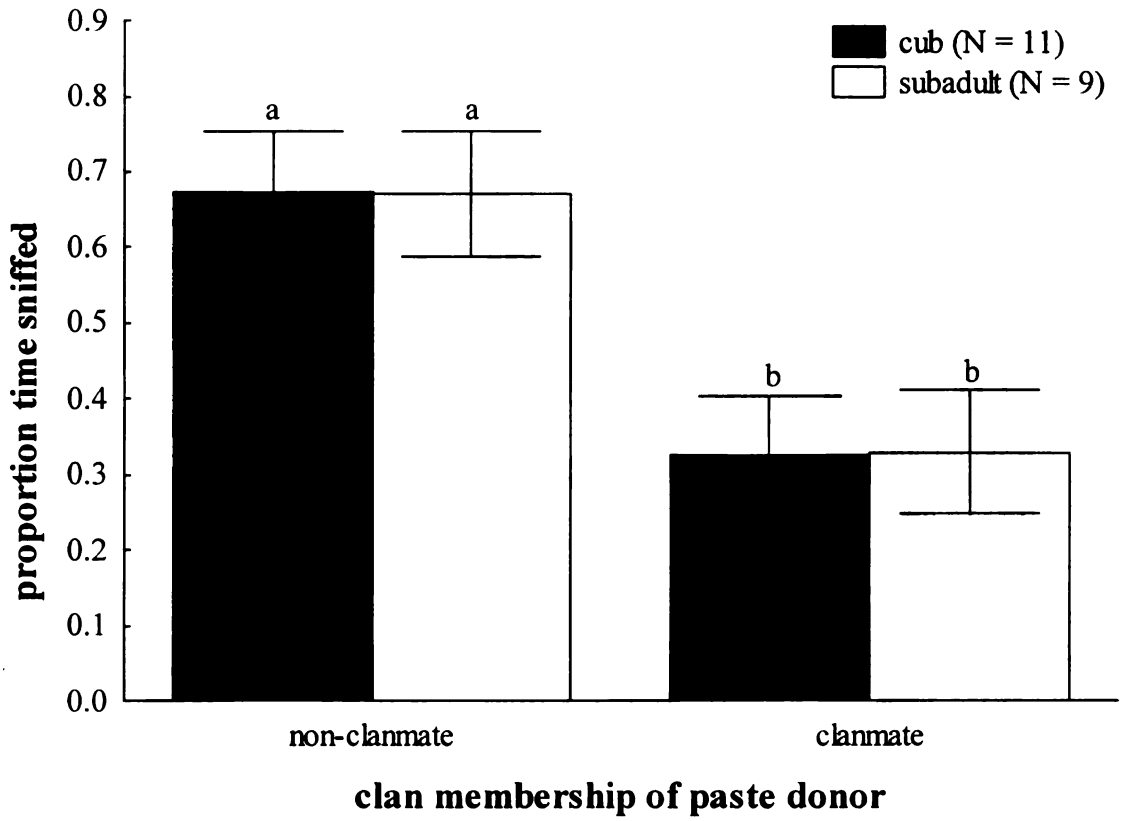



Figure 3.4. The relative amount of time cub and subadult spotted hyenas spent sniffing paste samples from female non-clanmate and female clanmate donors. Letters above the error bars indicate statistically significant differences ($P < 0.05$ (one-sided)).


Figure 3.5. The relative amount of time cub, subadult and adult female spotted hyenas spent sniffing paste samples from members of neighboring clans, distant clans, and control wires. "n.s." indicates a statistically non-significant difference between time spent sniffing odors from neighboring and distant clan members, following a significant overall treatment effect for that age class ($P < 0.05$). Sample sizes for cubs, subadults and adult females are 12, 10 and 5, respectively.

stimulus at least once. They did not differentially overmark the scent of neighboring and distant clan members, or the control wires (Friedman ANOVA, $\chi^2_{6,2} = 1.60$, P = 0.449).

**Distant clan vs. distant clan**

Given that this experiment was conducted exclusively on a single clan, we did not obtain sufficient sample sizes for examining differences in response among participants based on their age / sex class. Therefore, for this experiment all participants, excluding immigrant males, were analyzed collectively. There was a significant effect of treatment (Friedman ANOVA, N = 8, $\chi^2_{8,2} = 7.16$, P = 0.028; Figure 3.6), but participants did not significantly differ in the relative lengths of time they sniffed paste samples from West Talek and East Talek hyenas (Wilcoxon matched pairs, $T = 8.00$, P = 0.195). The lone immigrant male to participate in this experiment investigated the samples from West Talek and East Talek hyenas for the same amount of time. Four juveniles (2 cubs, 2 subadults) overmarked an experimental stimulus at least once. There were no apparent patterns in their overmarking responses.

**Male vs. female**

Neither cubs (Wilcoxon matched pairs, N = 12, $T = 36.00$, P = 0.850), nor subadults (N = 11, $T = 18.00$, P = 0.206), differentially investigated paste samples from male and female donors (Figure 3.7). Among subadult participants, males and females did not significantly differ in their responses to male and female odors (Mann-Whitney $U$, $N_{male} = 5$, $N_{female} = 6$, $U = 7.00$, P = 0.177). Unlike juveniles, adult females spent

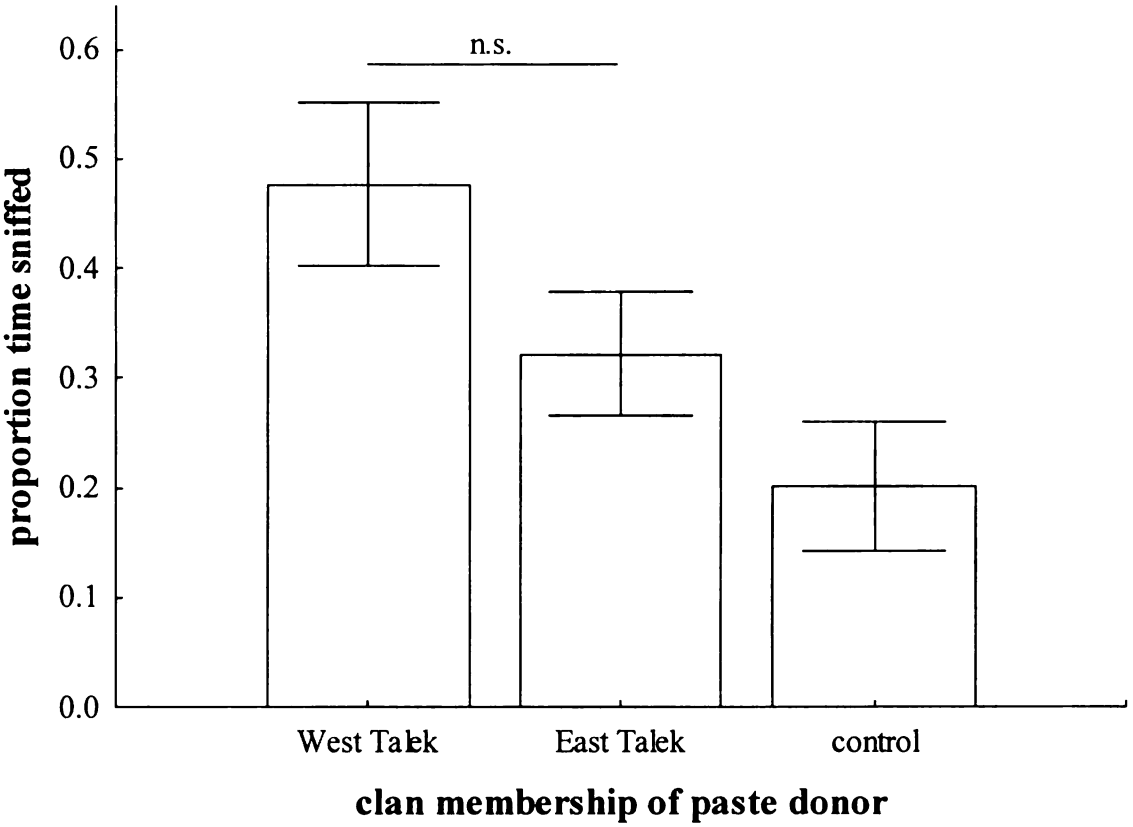



Figure 3.6. The relative amount of time spotted hyenas spent sniffing paste samples from members of the West Talek clan, East Talek clan, and control wires. Both the West and East Talek clans' territories were separated from the test clan's territory by at least one other clan. "n.s." indicates a statistically non-significant effect of clan membership, following a significant overall treatment effect ($P < 0.05$, $N = 8$).

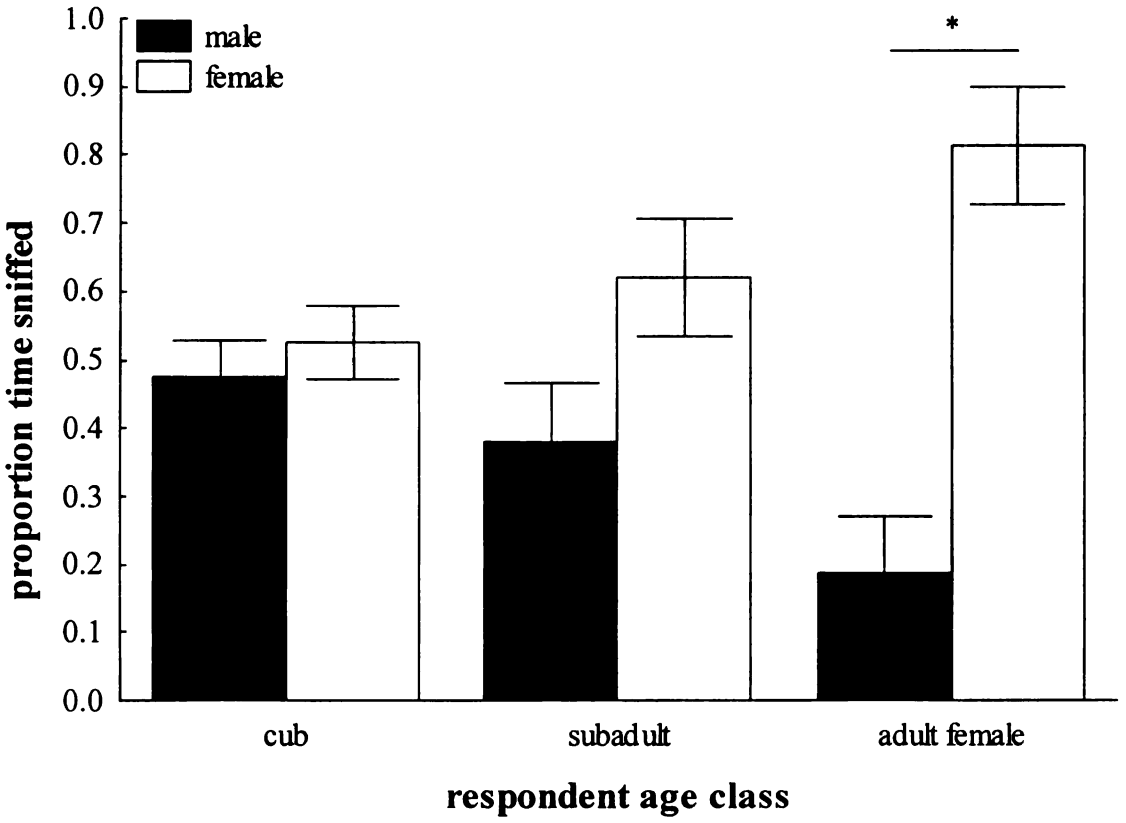



Figure 3.7. The relative amount of time cub, subadult and adult female spotted hyenas spent sniffing paste samples from adult male and female donors. The asterisk indicates a statistically significant difference ($P < 0.05$). Sample sizes for cubs, subadults and adult females are 12, 11 and 7, respectively.

substantially more time sniffing paste samples from female than male donors (N = 7, $T$ = 1.00, P = 0.031; Figure 3.7). Due to low sample size, we could not statistically evaluate whether adult females made this discrimination prior to any overmarking of experimental stimuli. However, each of the four females that did investigate stimuli before any overmarking occurred investigated paste from female donors more than paste from male donors. A single immigrant male participated in this experiment and he spent three times as much time sniffing paste from females as from males.

Eleven juveniles (6 cubs, 5 subadults) overmarked an experimental stimulus at least once. They did not overmark paste samples from one sex more than the other ($T$ = 17.50, P = 0.945). There was also a single instance of overmarking by an adult female. This female overmarked a paste sample obtained from an adult male residing in a distant clan. Interestingly, she was pregnant when she participated in this trial, as she gave birth to a litter 77 days later.

**Pregnant vs. late lactating**

Sample sizes were insufficient for us to evaluate effects of treatment among different age classes separately ($N_{cub}$ = 1, $N_{subadult}$ = 6, $N_{adult\ female}$ = 3). Considered collectively, however, natal spotted hyenas spent substantially more time sniffing paste samples from pregnant females than females sampled in late lactation (N = 10, $T$ = 0.00, P = 0.002; Figure 3.8). The effect of donor reproductive state on relative investigation time was apparent when limiting the analysis to responses that occurred before any hyenas overmarked the experimental stimuli (N = 6, $T$ = 0.00, P = 0.031). Three immigrant males participated in this experiment. Two of them exclusively sniffed paste

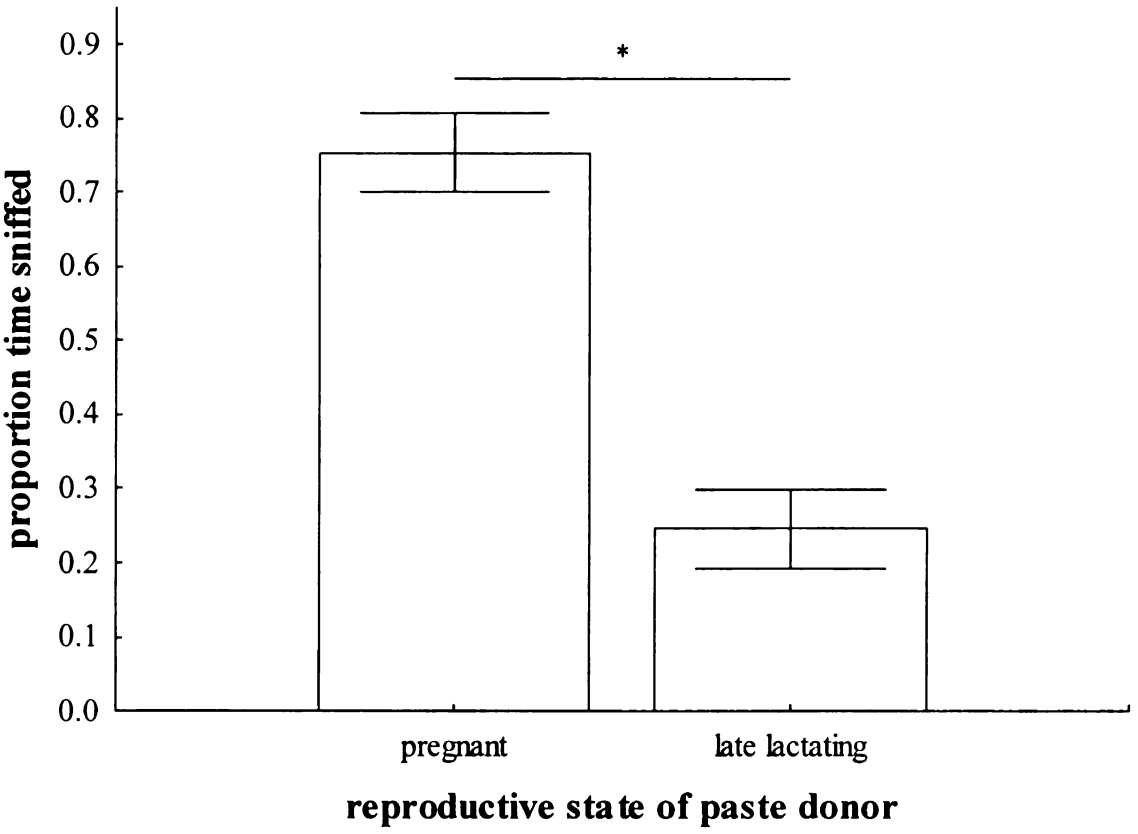



Figure 3.8. The relative amount of time natal spotted hyenas spent sniffing paste samples from pregnant and late lactating female donors. The asterisk indicates a statistically significant difference ($P < 0.05$, $N = 10$).

samples from late lactating females. The third investigated scent from a pregnant female for a slightly longer period of time than it did scent from a late lactating female. A single subadult male overmarked stimuli during this experiment, overmarking samples from pregnant female donors twice.

## DISCUSSION

This study was the first systematic investigation of the discrimination of conspecific scent by free-living spotted hyenas. It was also the first investigation of the scent discrimination abilities of hyenas that included cub, subadult and adult female respondents. Drea et al. (2002) showed that captive adult hyenas discriminated between paste samples from unfamiliar and familiar individuals, as well as between samples from male and female donors. Here we have extended these findings by showing that free-living hyenas discriminated between paste samples from female clanmates and non-clanmates, males and females, and pregnant and late lactating female donors.

### Clanmate vs. non-clanmate

The efficient and accurate recognition of group members is a critical component of territorial behavior in gregarious species (Brennan & Kendrick 2006). Several social mammals have been shown to recognize group members on the basis of scent alone (e.g. O'Riain & Jarvis 1997; Muller & Manser 2007; Palphramand & White 2007). Although captive spotted hyenas have been shown to discriminate between paste samples from familiar and unfamiliar donors (Drea et al. 2002), and hyenas have been observed reacting aversively to alien scent marks encountered in their territories (Kruuk 1972;

Mills 1990), prior to the current study, no one had yet systematically determined whether free-living hyenas discriminate between clanmates and non-clanmates on the basis of scent alone. Here we have demonstrated that juvenile hyenas clearly discriminated between paste samples from female clanmates and non-clanmates. Interestingly, juveniles did not discriminate between paste samples from male clanmates and non-clanmates. This may be a reflection of the labile nature of adult male membership in hyena clans, as compared to that of philopatric females (Frank 1986a).

Although juvenile hyenas differentially investigated paste samples based on clan membership, adult females did not. Considering that adult female spotted hyenas assume primary roles in territorial advertisement and defense (Boydston et al. 2001), we had expected adult females to spend considerably more time sniffing the scents of non-clanmates (Kruuk 1972; Mills 1990), especially those from female donors. Scent marks from alien female donors represent potential threats to territorial security, whereas scent marks from alien males likely do not. As is typical among mammals, spotted hyenas exhibit male-biased dispersal (Smale et al. 1997) and, therefore, the appearance of transient males within a clan's territory is a relatively common occurrence.

A challenge associated with scent discrimination studies is that, although positive experimental results indicate discriminative ability, negative results do not necessarily indicate *in*ability to discriminate (Brown 1979). Instead, negative results may be a product of insufficiently motivated participants, underlying contextual factors, or the scent samples from different donor groups being equally informative and biologically relevant (Brown 1979; White et al. 2004). In a previous study (Theis et al. *submitted*), we suggested that, for adult female hyenas, pasting functions to facilitate social cohesion

within groups. It is therefore possible that, for adult female hyenas, monitoring the presence and condition of same-sex competitors, whether they are clanmates or non-clanmates, is equally important. Certainly, more data on the responses of adult female hyenas to scents from clanmate and non-clanmate female donors are needed.

**Neighboring clan vs. distant clan**

If social animals discriminate between odors from members of their own social group and others, as juvenile hyenas in this study have shown they do, their discriminative ability may be based on the degree of familiarity they have with donors or instead on their recognition of chemical badges of group membership. Among free-living animals, individuals are more familiar with their neighbors than they are with strangers that reside farther away (see Muller & Manser 2007). Therefore, if discrimination between the scents of group members and others is based on degrees of familiarity, then free-living animals should discriminate between odors from neighbors and strangers. Indeed, it has been shown that members of multiple mammalian species make this discrimination (e.g. Sliwa & Richardson 1998; Rosell & Bjorkoyli 2002; Muller & Manser 2007; Palphramand & White 2007). In general, mammals that make this discrimination spend more time investigating scent marks from strangers than neighbors (e.g. Sliwa & Richardson 1998; Rosell & Bjorkoyli 2002; Palphramand & White 2007), because unfamiliar individuals typically pose a greater threat to territorial maintenance than do established neighbors ('dear enemy' effect: Fisher 1954; Temeles 1994). However, among territorial social mammals, this is not necessarily the case (Muller & Manser 2007). For highly gregarious mammals, the scent mark of a single transient

stranger within their territory likely represents less of a threat to a social group than does the scent mark of a member of a neighboring social group placed beyond an established border ('nasty neighbor' effect: Muller & Manser 2007). To date, very few studies have considered whether highly social mammals discriminate between the scent marks of neighbors and members of distant social groups and, if so, whether they exhibit greater interest in scents from their neighbors (e.g. Muller & Manser 2007).

Drea et al. (2002) demonstrated earlier that captive adult spotted hyenas discriminated between pastes samples from familiar and unfamiliar donors. In the current study, there was insufficient participation by adult females for us to evaluate whether they discriminated between paste samples from members of neighboring and distant clans. However, four of the five adult female participants sniffed paste samples from members of distant clans more than they did samples from members of neighboring clans or the control wires. These data, while preliminary, suggest that adult hyenas discriminate between the scents of neighbors and strangers, and that a dear enemy effect may be operating even in this highly gregarious species.

Although few adult females participated in this experiment, several cubs and subadults did participate. Despite suggestive trends in the data, neither cubs nor subadults clearly discriminated between paste samples from neighboring and distant clans. It was unsurprising that cubs did not differentially respond to scents from neighboring and distant clans, as they should be equally unfamiliar with both. Hyena cubs are primarily confined to their clan's communal den (Holekamp & Smale 1998) and, since alien hyenas are hardly ever observed at communal dens (personal observation), cubs are unlikely to be familiar with the scents of any hyena that is not a clanmate. The similarity in the

responses exhibited by cubs and subadults was moderately surprising, however, as subadult hyenas do travel, feed and rest beyond the confines of the communal den within their clan's territory (Holekamp & Smale 1998). Older subadults have even been observed participating in border patrols (Theis et al. 2008). During a border patrol members of a hyena clan cooperatively investigate and demarcate one of their territorial boundaries with scent marks (Kruuk 1972; Henschel & Skinner 1991; Boydston et al. 2001). Participating in border patrols would likely increase subadult familiarity with the scent marks of their neighbors. Additionally, male hyenas begin engaging in predispersal forays from their natal clans at approximately 18 -24 months of age (Smale et al. 1997). During their forays into neighboring territories, male hyenas investigate the odors they encounter, presumably including the scent marks of the hyenas that reside there (Smale et al. 1997). Consequently, predispersal exploratory forays would increase a male subadult's familiarity with the odors of its neighbors, and potentially with the odors of members of distant clans as well. Therefore, responses to conspecific scents by subadult hyenas may be substantially influenced by experience, and the lack of discriminative ability exhibited by younger (12 – 18 months of age; 5/10 subadult respondents) and older (18 – 30 months of age) subadult hyenas in this experiment should be interpreted with caution.

**Distant clan vs. distant clan**

Social animals that discriminate between odors from members of their own social group and others, may do so based on their degree of familiarity with donors, or instead based on chemical badges of group membership. Chemical badges of group membership are available when the odors associated with individuals in the same social group are

more similar than the odors associated with members of different social groups. Group-specific odors have been identified in social insect species (e.g. vander Meer et al. 1989; Ruther et al. 2002), as well as in several gregarious mammals (e.g. O'Riain & Jarvis 1997; Bloss et al. 2002; Buesching et al. 2003; Burgener et al. 2008). Group-specific odors are believed to promote cohesion within social groups by facilitating accurate recognition among group members (Ruther et al. 2002; Buesching et al. 2003). For instance, among gregarious naked mole-rats (*Heterocephalus glaber*), being recognized as a group member substantially reduces the amount of aggression individuals receive from their social partners (O'Riain & Jarvis 1997).

Group-specific odors seemingly exist in the spotted hyena as well: the profiles of volatile carboxylic acids emanating from paste samples are more similar within than between hyena clans (Hofer et al. 2001; Burgener et al. 2008). Prior to the current study, however, no one had attempted to determine whether hyenas discriminated between paste samples obtained from members of two different clans. Our analyses failed to demonstrate that they do, but two confounding issues should be discussed. First, there was only modest participation in this experiment, with half (4/8) of the respondents being cubs. It is possible that cubs lack the experience or the motivation to discriminate between paste samples from members of two clans with which they are entirely unfamiliar. We mention this because each of the subadult (2) and adult female (2) participants sniffed paste samples from West Talek donors more than they did samples from East Talek donors. A second potentially confounding issue is that, when this experiment was conducted, we thought that the original Talek clan had fissioned into the East and West Talek clans by 2001. Therefore, we used paste samples collected in 2001

or later. However, recent analyses of the association patterns of hyenas in these clans suggest that the two Talek clans did not completely split until 2002 (J.E. Smith, Michigan State University, unpublished data). Since almost half (5/12) of the samples we used in this experiment were collected in 2001, the samples may not truly reflect group-specific characteristics.

**Male vs. female**

Chemical communication plays a critical role in the reproductive biology of most mammals (Eisenberg & Kleiman 1972; Johnson 1973; Johansson & Jones 2007). Even among mammals that appear highly sexually monomorphic, the odors produced by the two sexes are usually substantially dimorphic (Blaustein 1981). Given the importance of opposite-sex odors in mammalian reproductive biology, it is surprising that few studies have considered when during development mammals begin to discriminate between male and female odors (White et al. 2004). Here we have demonstrated that adult female, but not cub or subadult, spotted hyenas discriminate between paste samples from male and female donors. As noted above, in discrimination experiments, negative results do not necessarily mean that participants cannot discriminate between scent sources, but rather only that they do not do so (Brown 1979). The lack of discrimination between male and female odors exhibited by juvenile hyenas may reflect the absence of a functional role for such a discrimination in the social lives of young hyenas (White et al. 2004). The primary challenges for hyena cubs are being recognized as members of their social groups, gathering information about their clanmates, and becoming assimilated into their clan's dominance hierarchy (Holekamp & Smale 1998). Scent marks from unfamiliar

individuals may provide cubs with important information about their social environments, regardless of whether the marks were deposited by male or female donors (Holekamp & Smale 1993; Smale et al. 1993). For subadult hyenas, the primary challenge is independently obtaining food following weaning (Holekamp et al. 1997; Holekamp & Smale 1998). For subadult hyenas then, there is also no apparent functional role for differentially investigating male and female odors.

Among adult hyenas, however, a primary challenge is obviously maximizing one's reproductive potential (Holekamp et al. 1996; Holekamp & Smale 1998; Engh et al. 2002). In general, if adults investigate the chemical signals of opposite-sex individuals more than those of same-sex individuals, the signals under investigation are believed to function more in reproduction than in intrasexual competition or cooperation (White et al. 2004). For example, upon attaining reproductive maturity, female mice (*Mus musculus*) switch from avoiding lures scented by adult males to exhibiting a marked preference for them, presumably thereby facilitating reproduction (Drickamer & Brown 1998). In their study of captive spotted hyenas, Drea et al. (2002) found that adult females investigated paste samples from male donors more than those from female donors. Accordingly, the authors suggested that pasting likely has a reproductive function for spotted hyenas. In contrast, in the current study, we found that free-living adult female hyenas spent far more time investigating paste samples from female than male donors. Rather than supporting a reproductive function for pasting by spotted hyenas, our analysis suggests that pasting functions in intrasexual competition among female hyenas. A potential explanation for the difference between the results obtained in these two studies is that Drea et al. (2002) used captive animals while we utilized free-living subjects. The

importance and potential influence of context on the outcome of discrimination studies has been widely recognized (e.g. Eisenberg & Kleiman 1972; Brown 1979; Leger 1993), and it has been recommended that, if the logistic challenges associated with field experiments can be overcome, investigators should conduct their scent discrimination experiments in the field with free-living participants (Muller-Schwarze 2001).

Our finding that free-living adult female hyenas spent more time investigating odors from alien females than from alien males is consistent with results obtained in a study of female ringtailed lemurs (*Lemur catta*; Scordato & Drea 2007). Female ringtailed lemurs consistently investigated odors from extra-group females more than odors from extra-group males, suggesting that scent marking by female lemurs functions in intrasexual competition and resource defense (Scordato & Drea 2007). The degree of interest female lemurs showed toward the odors of other females may be due to their living in female-dominated societies (Scordato & Drea 2007). The results of the current study lend support to this hypothesis, as spotted hyenas also live in female-dominated societies (Kruuk 1972; Frank 1986b; Mills 1990). However, the results of both studies may be more broadly indicative of patterns that exist among highly social female mammals in general, at least among those that participate in resource defense. In the current study, female hyenas were responding to the presence, inside their clan's territory, of scent marks from alien males and females. As noted above, hyenas exhibit male-biased dispersal (Frank 1986a; Smale et al. 1997) and, therefore, the appearance of a transient male inside a clan's territory is a common occurrence. However, since female hyenas are philopatric (Frank 1986a), and highly territorial (Kruuk 1972; Mills 1990; Boydston et al. 2001), the appearance of an alien female in a clan's territory is much less common (but

see Hofer & East 1993b). Additionally, the presence of alien females or their scent marks are potentially of great consequence to adult female hyenas because they may indicate intrusion pressure. Among social species in which females participate in resource defense, females likely benefit from actively monitoring the presence and condition of same-sex, extra-group competitors, as this information potentially serves to modulate interactions between social groups (Wittemyer & Getz 2007; Meyer et al. 2008).

**Pregnant vs. late lactating**

In many mammals, including social species (e.g. Scordato & Drea 2007; Meyer et al. 2008), females advertise their reproductive condition and sexual receptivity via scent marking (Johnson 1973; Johansson & Jones 2007). In doing so, they can facilitate mating and conception, and potentially reduce the amount of harassment they receive from male conspecifics during periods when they are not receptive (Johansson & Jones 2007). Here we simultaneously presented hyenas with paste samples from pregnant females and females in late lactation, to determine whether pasting by female hyenas advertises their sexual receptivity. As sexual receptivity among female hyenas typically occurs during late lactation (Szykman et al. 2003), if females advertise their receptivity via pasting, adult males would be expected to exhibit greater interest in scents from late lactating than pregnant female donors. Unfortunately, only three adult males participated in this experiment and their responses to the stimuli varied. Therefore, we could not evaluate this prediction.

Although we could not characterize the responses of males collectively, each of the other participants in this experiment investigated paste samples from pregnant

females more than those from late lactating donors. Therefore, paste from female donors clearly contains information about their reproductive condition, and natal animals exhibited a particular interest in the scents of pregnant females. Interestingly, in a previous study (Theis et al. *submitted*), we found that adult female hyenas pasted substantially more often during pregnancy than they did at other times. Although we do not yet clearly understand how female hyenas benefit from communicating information about their pregnancies, studies in other mammals have provided clues about the types of information they may be broadcasting. The urine of pregnant female mammals contains information about the fetuses females carry, specifically their paternal haplotypes at the major histocompatibility locus (Beauchamp et al. 1994; Beauchamp et al. 1995). In other words, odorants in a pregnant female's urine provide receivers with the identity of her offspring's sire. These findings are pertinent to the current study because this information is not necessarily available only in urine, rather it may additionally be available in other female secretions, including saliva, milk and sweat (Beauchamp et al 1995; Beauchamp et al. 2005). It is possible that this information is available in the paste secretions of pregnant female hyenas as well.

For female mice, communicating information about an offspring's paternal haplotype may reduce the likelihood of that offspring becoming a victim of infanticide (Beauchamp et al. 2005). Additionally, in being pre-exposed to the fetal odortypes of their offspring, females may be more committed to providing maternal care after giving birth (Beauchamp et al. 2005). We find it intriguing to expand consideration of this idea from the mother alone to the entire social group. A primary challenge for newborn social carnivores is being effectively recognized as members of their social groups, thereby

reducing the amount of aggression they receive from their social partners (Rasa 1973; Fell et al. 2006). If paste from pregnant female hyenas contains fetal odortypes, then increased rates of pasting by females during pregnancy might serve to familiarize clanmates with the odors of their offspring prior to their being born. This could potentially facilitate the recognition and assimilation of their offspring into the clan.

**Future directions**

As appropriate samples become available, some of our experiments should be replicated to bolster sample sizes and increase the resolution of response patterns among different age / sex classes. The protocols used in the current study were effective and are simple enough that the experiments can be readily replicated. However, a minor adjustment to the protocols should be made to facilitate participation by adult hyenas, especially immigrant males, in the experiments. The location of experiments should be moved from the immediate vicinity of communal dens to alongside well-traveled trails that hyenas use when arriving at these dens. Although this adjustment will be labor intensive, it has two substantial benefits. First, by moving the experimental stimuli away from the immediate vicinity of dens, juvenile hyenas are less likely to engage the stimuli before adults have the opportunity to do so. This will remedy the potentially confounding issue of juveniles overmarking the stimuli. Second, since immigrant males in attendance at communal dens remain at the peripheries of den areas (East & Hofer 1991, 1995; White 2007), setting up experiments beside primary paths leading to dens will increase the likelihood of adult males encountering and interacting with the stimuli. If sample sizes of adult respondents can be bolstered in all experiments, then we will be better able

to consider how the reproductive condition (White et al. 2004; Rasmussen et al. 2005; Scordato & Drea 2007; Palphramand & White 2007) and social status (Rostain et al. 2004) of experimental participants influences their responses to conspecific scents.

Although the current study has further developed our understanding of the information content of paste, and consequently improved our understanding of the functions of pasting behavior among spotted hyenas, there is likely additional information available in paste as well. Specifically, paste may provide receivers with information about the donor's age (White et al. 2003), health (Zala et al. 2004), diet (Ferkin et al. 1997), or social status (Hayes et al. 2001). In the future, it would be beneficial to conduct additional scent discrimination experiments with free-living hyenas to discern whether this information is available in paste as well.

CHAPTER FOUR

# A BACTERIAL MECHANISM FOR GROUP-SPECIFIC ODORS IN THE SPOTTED HYENA (*CROCUTA CROCUTA*)

## INTRODUCTION

Animal bodies contain substantially more bacterial than animal cells (Savage 1977). Many of these symbiotic bacteria are not pathogenic to their hosts; rather they are mutualists, some being absolutely necessary for the proper development and functioning of animal bodies (Dethlefsen et al. 2007; Cogen et al. 2008). These realizations have catalyzed numerous investigations of the effects of symbiotic bacteria on animal development, physiology, and health (e.g. Rawls et al. 2004; Dethlefsen et al. 2006; Haine 2008), but few studies have yet addressed the potential roles that bacteria might play in animal behavior.

Animal behavior is a dynamic comprehensive field of study that synthesizes material from other fields of biological study, including evolution, genetics, development, physiology, neurobiology, endocrinology and psychology (Dugatkin 2004). However, one field remains glaringly outside the current scope of animal behavior: microbial ecology. Within two widely used contemporary textbooks of animal behavior, there exists only a single discussion of bacteria in each (Dugatkin 2004; Alcock 2005). In both cases, the discussion is of behavioral adaptations animals have evolved to avoid being infected by pathogenic bacteria. A lack of consideration of bacteria in animal behavior research is evident in the contemporary primary literature as well. A recent Web of Science keyword search (bacteria*) of six peer-reviewed animal behavior research journals yielded only fifteen publications addressing bacteria since the year 2000

(http://apps.isiknowledge.com; accessed 08/15/08; *Animal Behaviour, Behavioral Ecology, Behavioral Ecology and Sociobiology, Behaviour, Ethology, Journal of Ethology*). Only three of these fifteen publications discussed potential beneficial associations between bacterial symbionts and their animal hosts (Buesching et al. 2003; Lam et al. 2007; Lombardo 2008).

One aspect of animal behavior in which bacteria likely figure prominently is communication (Albone 1984; Muller-Schwarze 2006). For most animals, communication is achieved largely via chemical means (Wyatt 2003). The components of chemical signals may be obtained directly from signalers' diets, synthesized by signalers themselves, or generated as by-products of symbiotic bacterial metabolism (Gorman & Trowbridge 1989). Many mammals communicate chemically by scent marking with secretions from specialized scent glands (Albone 1984; Wyatt 2003; Muller-Schwarze 2006). Mammalian scent glands are warm, moist, anaerobic, organic-rich, and thus highly conducive to the proliferation of symbiotic bacteria (Albone 1984; Gorman & Trowbridge 1989). When symbiotic bacteria ferment the organic-rich sebum substrates within these glands, they generate volatile fatty acid by-products (Albone et al. 1974; Gorman et al. 1974), and these fatty acids seem to be prominent and active components of mammalian scent marks (e.g. Gorman 1976). Consequently, it has been postulated that symbiotic bacteria are critically involved in the production of social odors among mammals that engage in scent marking (Albone et al. 1974; Gorman 1976). All else being equal (age, sex, reproductive state, etc.), if conspecifics harbor unique communities of bacteria within their scent glands, then they should emit individually distinct signature odors via scent marking (Gorman 1976). Alternatively, if they harbor similar microbial

communities in their scent glands, then they should emit similar odors (Albone et al. 1974; Macdonald 1985). Members of mammalian social groups may come to share similar microbial communities in their scent glands, and thus emit group-specific odors, as a result of occupying the same physical space, coming into frequent bodily contact with one another, or repeatedly scent marking the same sites (Albone et al. 1974; Macdonald 1985; Buesching et al. 2003). At the functional level, group-specific odors are believed to facilitate recognition among group members, thereby reducing levels of aggression among social partners and promoting social cohesion within groups (Albone et al. 1974; O'riain & Jarvis 1997; Bloss et al. 2002; Safi & Kerth 2003; Fell et al. 2006)

If the hypothesis were correct that symbiotic bacteria are responsible for group-specific social odors in mammals, then the structure of bacterial communities in the scent glands of members of the same social group should be more similar than the structure of communities found in members of different groups. This prediction was previously tested using captive red foxes (*Vulpes vulpes*), and the results did not support the hypothesis (Albone et al. 1978). However, as the authors of the study themselves noted, their culture-based approach to sampling bacterial communities was likely biased due to the differential recoverability of anaerobic bacterial forms from culture (Albone et al. 1978; Schloss & Handelsman 2004). At present, most bacteria remain unculturable, so culture-independent community sampling techniques, such as 16S rRNA gene surveys, provide valuable alternatives to traditional culture-based approaches (Riesenfeld et al. 2004; Dethlefsen et al. 2007). The 16S rRNA gene is critical in protein manufacture, and is therefore a universal and highly conserved gene that allows researchers to make taxonomic identifications of organisms based on their nucleotide sequences at this locus

(Dethlefsen et al. 2007). The objective of the current study was to re-evaluate the hypothesis that bacteria are responsible for the production of group-specific social odors in mammals, using free-living spotted hyenas (*Crocuta crocuta*) as subjects, and employing culture-independent 16S rRNA gene survey sampling techniques.

Spotted hyenas are large carnivores found throughout sub-Saharan Africa. They live in complex social groups, called clans, that typically consist of 40 - 80 individuals (Kruuk 1972; Trinkel et al. 2007). Members of each clan cooperatively defend their group's territory from neighboring hyenas (Kruuk 1972; Mills 1990; Henschel & Skinner 1991; Boydston et al. 2001). Hyena clans are fission-fusion societies, in which members are seldom all present in the same place at the same time; rather members fission into subgroups that change in composition and size several times each day (Holekamp et al. 1997; Smith et al. 2008). Hyena clans contain multiple breeding males and multiple overlapping generations of females. Genetic relatedness is not significantly greater within than among hyena clans, because of high levels of male-mediated gene flow among clans (Van Horn et al. 2004). In contrast to most mammalian social systems, hyena societies are female-dominated, and social status within a clan's linear dominance hierarchy is not determined by size or fighting ability (Frank 1986a,b). Instead, natal hyenas 'inherit' the social ranks of their mothers (Frank 1986b; Holekamp & Smale 1991, 1993; Smale et al. 1993; Engh et al. 2000), and adult immigrant males, all of which are subordinate to natal individuals, queue for dominance status with other immigrant males (Smale et al. 1997; East & Hofer 2001).

To mediate the complex social relationships between and within clans, spotted hyenas utilize a rich array of vocal, visual and chemical signaling behaviors (Kruuk 1972;

Mills 1990). A common and conspicuous form of chemical signaling among spotted hyenas is 'pasting.' When pasting, hyenas extrude their anal scent pouch and drag it over grass stalks. In doing so, hyenas typically deposit secretions near the tops of the grass stalks (Matthews 1939; Kruuk 1972; Mills 1990). These secretions, referred to as 'paste,' are produced in bi-lobed sebaceous glands that are separate from the gastrointestinal tract and empty their products into the anal pouch (Matthews 1939; Mills 1990). The paste of spotted hyenas is rich in fatty acids (Buglass et al. 1991; Hofer et al. 2001), and it has been shown that the chemical profiles of paste are group-specific (Hofer et al. 2001; Burgener et al. 2008). Specifically, the relative abundances of volatile odorants, primarily fatty acids, emanating from paste are more similar among clanmates than they are among members of different clans (Burgener et al. 2008). It has been suggested that the group-specific differences in the odor of paste may be due to the different metabolic activities of different communities of symbiotic bacteria within the scent glands of spotted hyenas (Burgener et al. 2008; Theis et al. 2008). The primary objective of the current study was to inquire whether group-specific bacteria may be responsible for the production of group-specific social odors in the spotted hyena. A secondary objective was to preliminarily describe the microbiome of the spotted hyena anal pouch.

## METHODS

### Study area and sample collection

This study was conducted in the Masai Mara National Reserve, Kenya. From 1999 - 2000, we obtained paste samples directly from the anal pouches of sixteen spotted hyenas anaesthetized with Telazol (W.A. Butler; 6.5 mg/kg) delivered from a $CO_2$-

powered darting rifle (Telinject). The sampled hyenas represented four distinct clans within the north-central part of the Reserve: Emarti Hill, Mara River, Fig Tree and Southern Comfort (Figure 4.1). We collected paste samples from four adult lactating females from each of these clans. Paste samples were placed in sterile cryogenic vials (Corning), stored in liquid nitrogen, and transported to Michigan State University, where they remained frozen (minimum -20 C) until being used in the current study.

**Construction of 16S ribosomal DNA clone libraries from paste samples**

We used a Mo Bio ultraclean fecal DNA kit to isolate DNA from each paste sample (~ 0.1 g of paste per sample). Each extraction was then diluted (1:10), and the 16S rDNA in each was amplified by polymerase chain reaction (PCR) using two bacterial primers (8F, 5' - AGA GTT TGA TCA TGG CTC AG - 3'; 1392R, 5' - ACG GGC GGT GTG TAC - 3'). The PCR program was: 95 C for 3 min, followed by 30 cycles consisting of 95 C for 30 sec, 55 C for 30 sec, 72 C for 45 sec, and a final extension period of 72 C for 10 min. Amplicons were then ligated into plasmid vectors (pCR 2.1-TOPO; Invitrogen) that were used to transform *Escherichia coli* (TOP10) and generate a 16S rDNA clone library for each paste sample. We randomly selected 47 clones from each library for DNA sequencing. High throughput sequencing was performed at the Research Technology Support Facility of Michigan State University, using the 8F primer.

Eighty nine percent (670 / 752) of the sequencing reactions yielded quality sequences (Geospiza Finch; Q>20) of typically 500 - 800 base pairs in length (at least 300 bp). These 670 sequences were aligned using the automated aligner in Arb software, and then adjusted by hand to comply with secondary structure models of the 16S rRNA

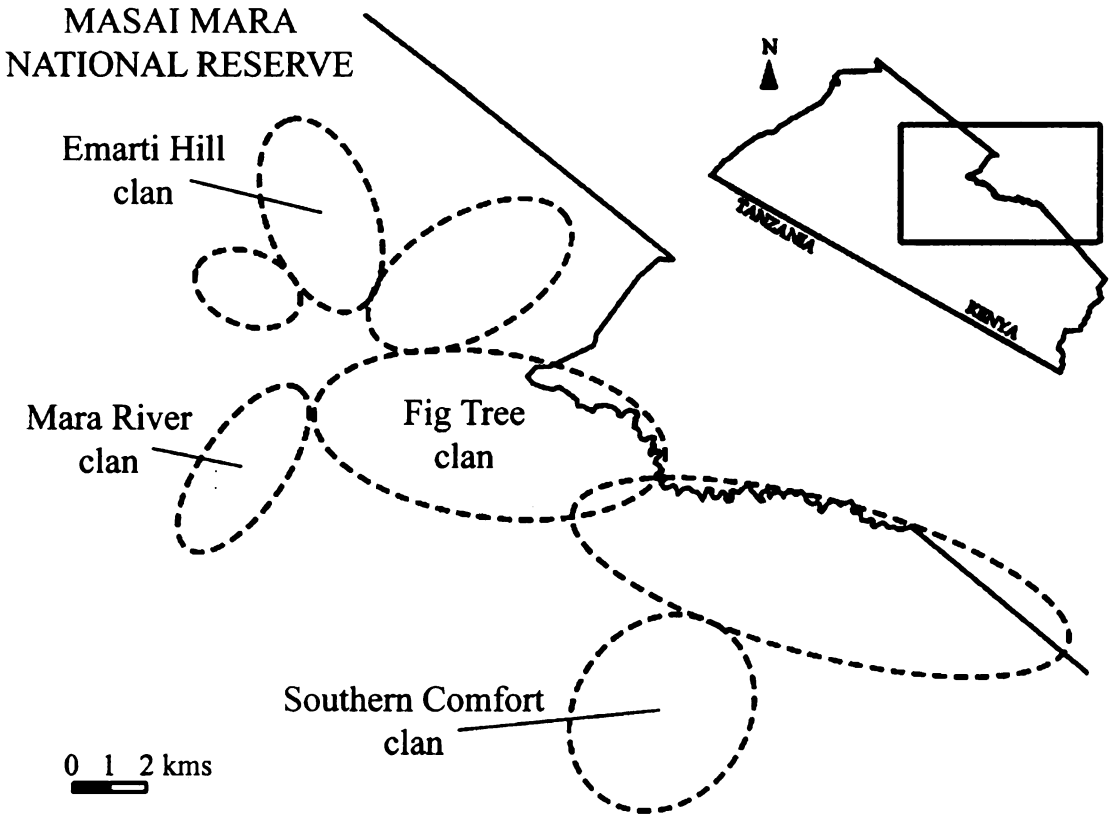



Figure 4.1. Map illustrating the relative locations within the Reserve of the four hyena clans sampled in this study. The dashed lines indicate the estimated territorial boundaries of hyena clans in this area of the Reserve from 1999 - 2000. This map was adapted with permission from Van Horn et al. (2004) and Kolowski et al. (2007).

gene (ssu_jan04 database release; Ludwig et al. 2004). The aligned sequences (specifically the region between base positions 60 and 651 of the 16S rRNA gene; *E. coli* numbering) were incorporated into a distance matrix, which was used to assess sequence similarities and assign sequences to operational taxonomic units (OTU) via the program DOTUR (average neighbor joining method; Schloss & Handelsman 2005). In the current study, OTUs were defined based upon a 97% sequence identity cutoff, and therefore represent approximate species-level associations (Stackebrandt & Goebel 1994).

**Describing the anal pouch microbiome**

From the DOTUR output, we generated rarefaction and rank abundance curves for all sequences considered collectively. Additionally, we recorded a Chao1 richness estimate and a Shannon diversity index (which considers evenness in addition to richness; Magurran 2004) for the symbiotic bacterial community associated with the anal pouch. Our purpose in constructing rarefaction and abundance curves and in calculating diversity measures was to evaluate our sample coverage.

*Species-level taxonomic identities of OTUs in the anal pouch microbiome*

To determine the species-level identities of bacteria comprising the anal pouch microbiome, we randomly selected a single representative sequence from each OTU, and submitted the representative sequences to the Silva incremental aligner (SINA; http://www.arb-silva.de/aligner; Pruesse et al. 2007). SINA aligned the representative sequences with their ten nearest neighbors in the Silva reference library. We then imported the representative sequences and their ten nearest neighbors into Arb and

generated another distance matrix. We imported this matrix into DOTUR, and determined whether the sequences representing the OTUs shared 97% sequence similarity with any of their nearest neighbors in the Silva reference library (Silva Release 95). If they did, and if the neighboring sequences were obtained from cultured or *Candidatus* bacterial specimens, then we assigned corresponding species-level taxonomic identities to the representative sequences and their OTUs. One sequence shared 97% sequence similarity with ten uncultured environmental samples. As it also might have shared 97% sequence similarity with a cultured bacterium that was not one of its ten closest neighbors in the reference library, the sequence was resubmitted to SINA and the process was duplicated using 40 nearest neighbors instead of ten. The sequence in question shared 97% sequence similarity with all 40 of its nearest neighbors in the reference library, and again the neighboring sequences were all obtained from uncultured bacteria. We then used the sequence matching function (Seqmatch) available from the Ribosomal Database Project (Release 10, update 4; Cole et al. 2007) to broadly identify the sequence as belonging to the genus *Cetobacterium*. We used Arb to generate a distance matrix from the sequence in question and several sequences obtained from *Cetobacterium* and *Fusobacterium* (sister genus to *Cetobacterium*) type specimens. The percent similarities among these sequences were determined via DOTUR.

*Approximate genus-level taxonomic identities of OTUs in the anal pouch microbiome*

We selected a random representative sequence from each OTU that made up more than 1% of all sequences analyzed in the current study. These sequences were aligned in SINA using their ten nearest neighbors in the Silva reference library. The representative

sequences and their ten nearest neighbors were imported into Arb, and a neighbor-joining phylogenetic tree was constructed illustrating the relationships among the sequences. From this tree, we inferred genus-level taxonomic identities for the abundant members of the anal pouch microbiome.

**Alpha diversity: diversity within communities**

To assess our sample coverage, we generated separate distance matrices in Arb from sequences comprising each of the clone libraries. We also used DOTUR to construct rarefaction and abundance curves, and to calculate richness and diversity measures, for each of the clone libraries.

**Beta diversity: diversity between communities**

From the DOTUR output assessing degrees of similarity among all sequences considered collectively, we tabulated the number of sequences obtained per OTU per clone library. We entered these sampling data into the program EstimateS (Colwell 2006), and calculated Bray-Curtis similarity indices among all 16 clone libraries. The Bray-Curtis similarity index is widely used in ecological studies to assess the biotic distinctness of sampling communities (Magurran 2004; Quinn & Keough 2002). Moreover, the Bray-Curtis index was the most appropriate beta diversity measure for the current study because it was the distance measure employed to demonstrate that hyena paste has group-specific odorant profiles (Burgener et al. 2008).

We used the program Mega4 (Tamura et al. 2007) to perform a hierarchical cluster analysis of the 16 clone libraries based upon Bray-Curtis indices. Cluster analyses

produce dendrograms that illustrate the degrees of difference among sampled communities. We constructed our dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA; e.g. Pardo & Armitage 1997), based upon Bray-Curtis dissimilarity indices (1 - Bray-Curtis). We used dissimilarity, as opposed to similarity, measures so that the branch lengths on the dendrogram would correspond to degrees of difference in community structure among the clone libraries. We additionally visualized similarities in community structure among clone libraries via non-metric multidimensional scaling (MDS) plots, based again on Bray-Curtis dissimilarity indices (Statistica, v6.1, 2002). In MDS plots, sampled communities that are dissimilar are placed far apart whereas those that are similar are placed close together (Quinn & Keough 2002; Gotelli & Ellison 2004).

In addition to visually exploring our data, we also statistically evaluated similarities in community structure among clone libraries. Analysis of similarity (ANOSIM) is a conservative non-parametric analysis that evaluates whether significant differences exist between groups based on a distance measure (Clarke 1993; Quinn & Keough 2002; Gotelli & Ellison 2004). We conducted an ANOSIM testing the prediction that the structure of bacterial communities in the anal pouches of spotted hyenas would be more similar within than between clans. We entered sampling data into the program PAST (version 1.82b; Hammer et al. 2001), and $\log_{10}(x + 1)$ transformed them. The ANOSIM included 10,000 permutations, and was based on Bray-Curtis indices with clan membership as the independent variable.

## RESULTS

### Anal pouch microbiome

At a 97% sequence identity cutoff, we found 75 OTUs in the microbiome of the spotted hyena anal pouch (Chao1 = 136.9 (103.7 - 208.3, 95% CI); Shannon = 2.50 (2.38 - 2.63)). Only eleven of the 75 OTUs shared 97% sequence identities with previously cultured or well-categorized bacterial species (Table 4.1). Collectively, these eleven OTUs represented only two percent (14 / 670) of the sequences analyzed in the current study. Although our sample coverage did not appear complete (Figure 4.2), it was representative, as the anal pouches of the 16 sampled spotted hyenas appeared to be dominated by fewer than ten bacterial species (Figure 4.3). Nine OTUs each represented more than one percent of all sequences analyzed. Collectively, these nine OTUs represented 85 percent (569 / 670) of the sequences analyzed in this study, and appear to be members of the genera *Peptostreptococcus*, *Corynebacterium*, *Propionibacterium*, and *Anaerococcus* (Figure 4.4).

### Alpha diversity

Within clone libraries, sample coverage was not complete (Figure 4.5). As alpha diversity measures are affected by sampling effort, the Chao1 and Shannon diversity measures for these anal pouch communities should be interpreted cautiously (Table 4.2; Magurran 2004). However, no differences in phylotype diversity among libraries from different clans were apparent and, although sampling was not complete, it did appear to be representative. A majority of libraries contained fewer than ten OTUs, and each library was dominated by just a few phylotypes (Figure 4.6). Therefore, at a 97%

Table 4.1. OTUs in the current study that shared at least 97% sequence similarities with previously cultured or well-categorized (i.e. *Candidatus*) bacterial species.

| Representative sequence for OTU | Total number of sequences in OTU | Species sharing 97% sequence identity with OTU |
|---|---|---|
| MaraRiver2_H04 | 2 | *Finegoldia magna* |
| MaraRiver3_C09 | 2 | *Corynebacterium amycolatum*<br>*C. hansenii*<br>*C. freneyi* |
| FigTree4_E02 | 2 | *Pantoea agglomerans*<br>*P. ananatis* |
| FigTree3_C10 | 1 | *Corynebacterium falsenii*<br>*C. auriscanis*<br>*C. resistens*<br>*C. urealyticum* |
| EmartiHill4_F06 | 1 | *Cryptosporangium japonicum*<br>*C. arvum*<br>*C. aurantiacum*<br>*C. minutisporangium* |
| FigTree4_E06 | 1 | *Pseudomonas putida*<br>*P. rhizosphaerae*<br>*P. gingeri* |
| SouthernComfort2_G04 | 1 | *Peptoniphilus olsenii* |
| MaraRiver1_C08 | 1 | *Candidatus Peptoniphilus massiliensis* |
| FigTree3_B03 | 1 | *Ignavigranum ruoffiae* |
| SouthernComfort3_A08 | 1 | *Segetibacter koreensis* |
| MaraRiver2_G01 | 1 | *Cetobacterium somerae* |

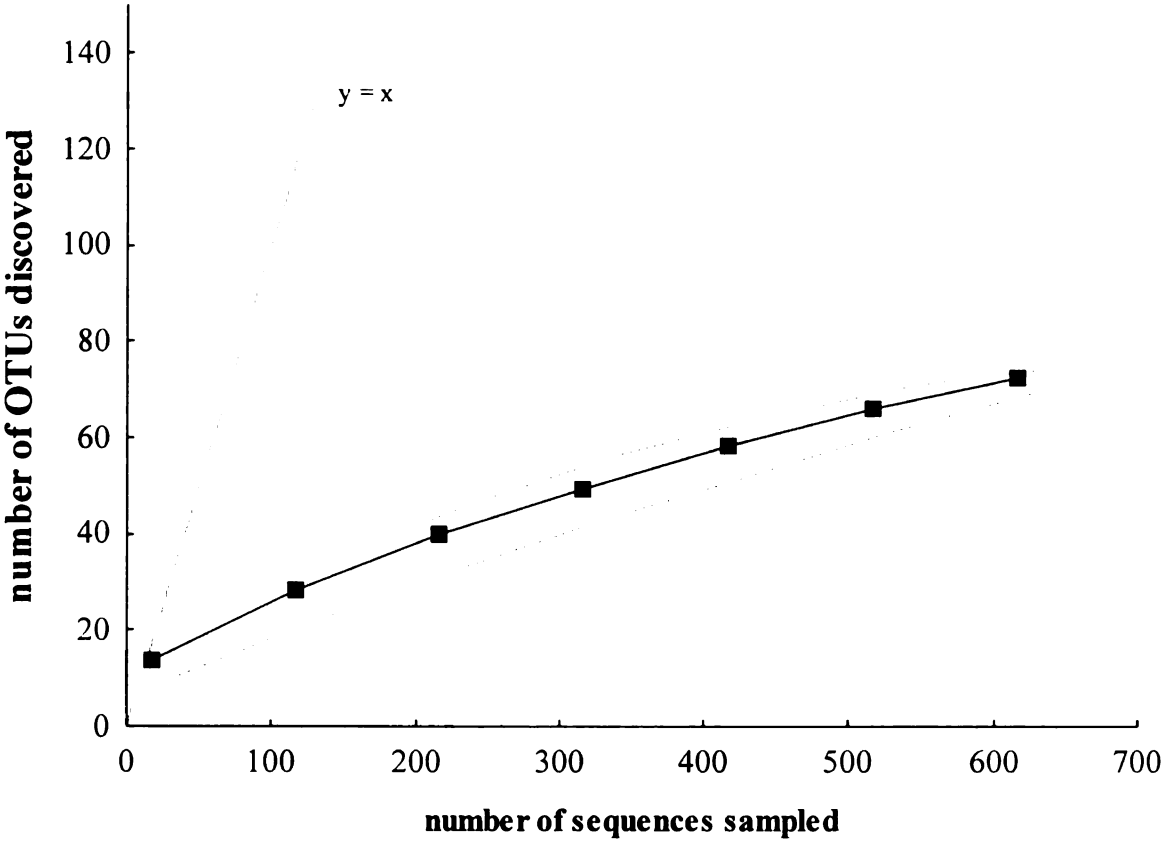



Figure 4.2. Rarefaction curve illustrating the degree of sample coverage in this study for all sampled hyenas considered collectively. The dotted curves represent the 95% confidence interval for the plot. To provide perspective when gauging the degree to which the rarefaction curve is leveling off, the function y = x is also illustrated.

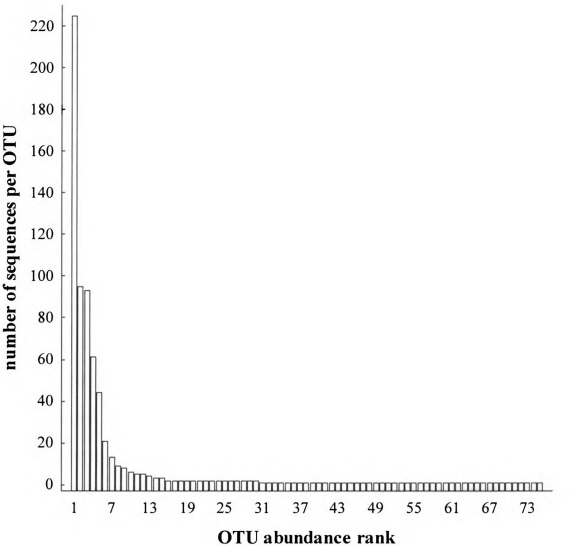
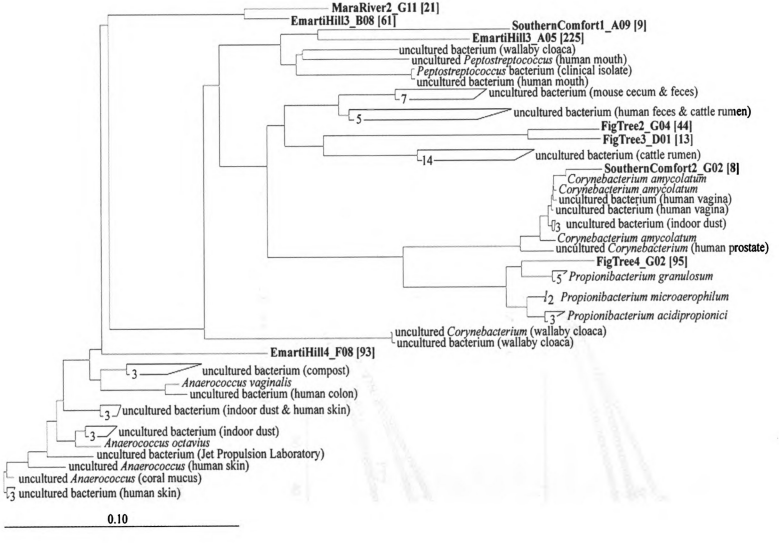



Figure 4.3. Rank abundance curve showing the relative abundance of the 75 OTUs found in this study.

Figure 4.4. Phylogenetic analysis of representatives of the nine OTUs (in bold face) that each represented more than 1% of all sequences analyzed in the current study. These nine representatives were incorporated into the phylogenetic tree with their ten nearest neighbors from the Silva reference library, as determined by SINA. With each representative sequence, the total number of sequences constituting that OTU is indicated in brackets. The numbers within branches indicate the number of nearest neighbor sequences that have been grouped together (i.e. condensed) on that branch. For nearest neighbor sequences obtained from uncultured bacteria, we have included information from the Silva database about the environmental source from which they were obtained.


MaraRiver2_G11 [21]
EmartiHill3_B08 [61]
SouthernComfort1_A09 [9]
EmartiHill3_A05 [225]
uncultured bacterium (wallaby cloaca)
uncultured Peptostreptococcus (human mouth)
Peptostreptococcus bacterium (clinical isolate)
uncultured bacterium (human mouth)
uncultured bacterium (mouse cecum & feces)
7
uncultured bacterium (human feces & cattle rumen)
5
FigTree2_G04 [44]
FigTree3_D01 [13]
14
uncultured bacterium (cattle rumen)
SouthernComfort2_G02 [8]
Corynebacterium amycolatum
Corynebacterium amycolatum
uncultured bacterium (human vagina)
uncultured bacterium (human vagina)
3
uncultured bacterium (indoor dust)
Corynebacterium amycolatum
uncultured Corynebacterium (human prostate)
FigTree4_G02 [95]
5
Propionibacterium granulosum
2
Propionibacterium microaerophilum
3
Propionibacterium acidipropionici
uncultured Corynebacterium (wallaby cloaca)
uncultured bacterium (wallaby cloaca)
EmartiHill4_F08 [93]
3
uncultured bacterium (compost)
Anaerococcus vaginalis
uncultured bacterium (human colon)
3
uncultured bacterium (indoor dust & human skin)
3
uncultured bacterium (indoor dust)
Anaerococcus octavius
uncultured bacterium (Jet Propulsion Laboratory)
uncultured Anaerococcus (human skin)
uncultured Anaerococcus (coral mucus)
3
uncultured bacterium (human skin)
0.10

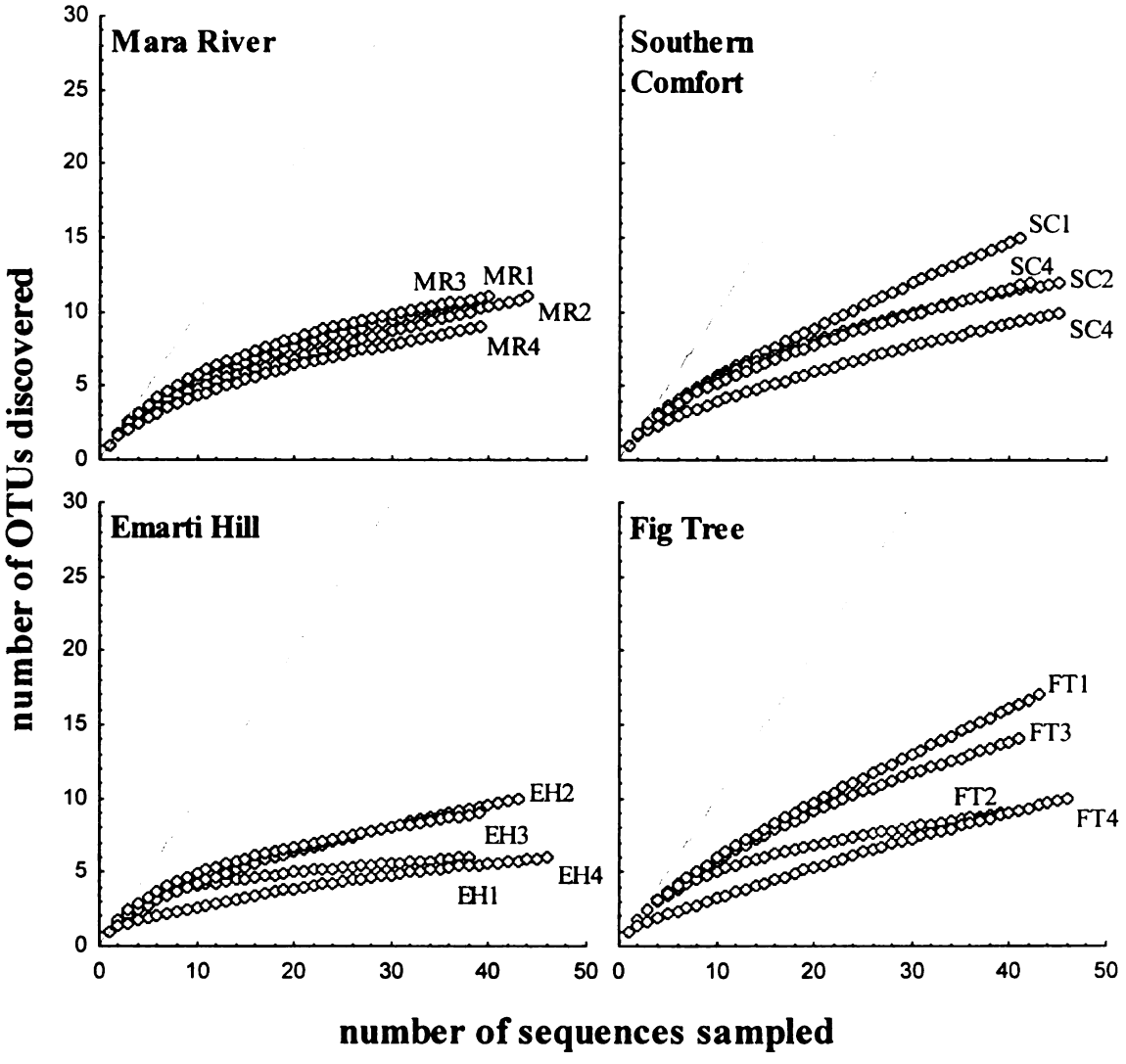



Figure 4.5. Rarefaction curves illustrating the degree of sample coverage for each hyena sampled in each clan. The clone libraries have been separated by clan of origin. The function y = x (dotted lines) appears on each graph for perspective.

Table 4.2. The number of sequences analyzed, and the number of OTUs found, in each clone library. Richness estimates and diversity indices of the bacterial communities within the anal pouches of the individual hyenas have been included, along with their 95% confidence intervals.

| Hyena | # seqs | OTUs | Chao1 richness estimate | Shannon diversity index |
|---|---|---|---|---|
| MR1 | 40 | 11 | 18.5 (12.3 - 53.5) | 1.85 (1.53 - 2.17) |
| MR2 | 44 | 12 | 21.5 (13.0 - 65.2) | 1.77 (1.46 - 2.08) |
| MR3 | 39 | 10 | 12.5 (11.2 - 23.5) | 2.00 (1.71 - 2.29) |
| MR4 | 39 | 10 | 19.0 (10.9 - 61.0) | 1.44 (1.06 - 1.82) |
| SC1 | 41 | 15 | 42.5 (21.7 - 128.5) | 2.05 (1.67 - 2.42) |
| SC2 | 45 | 12 | 14.5 (12.4 - 29.0) | 1.96 (1.65 - 2.26) |
| SC3 | 45 | 10 | 15.0 (10.9 - 38.9) | 1.40 (1.02 - 1.77) |
| SC4 | 42 | 12 | 19.0 (13.3 - 48.5) | 1.86 (1.52 - 2.20) |
| EH1 | 38 | 6 | 7.0 (6.1 - 19.7) | 1.43 (1.19 - 1.67) |
| EH2 | 43 | 10 | 17.5 (11.3 - 52.5) | 1.43 (1.05 - 1.82) |
| EH3 | 39 | 9 | 12.0 (9.4 - 32.0) | 1.73 (1.43 - 2.03) |
| EH4 | 46 | 6 | 7.5 (6.2 - 21.1) | 0.77 (0.43 - 1.12) |
| FT1 | 41 | 14 | 21.0 (15.5 - 47.7) | 2.08 (1.73 - 2.42) |
| FT2 | 39 | 9 | 15.0 (10.0 - 46.7) | 1.77 (1.49 - 2.05) |
| FT3 | 43 | 17 | 95.0 (44.2 - 240.5) | 2.12 (1.72 - 2.52) |
| FT4 | 46 | 10 | 17.0 (11.3 - 46.5) | 1.06 (0.64 - 1.48) |

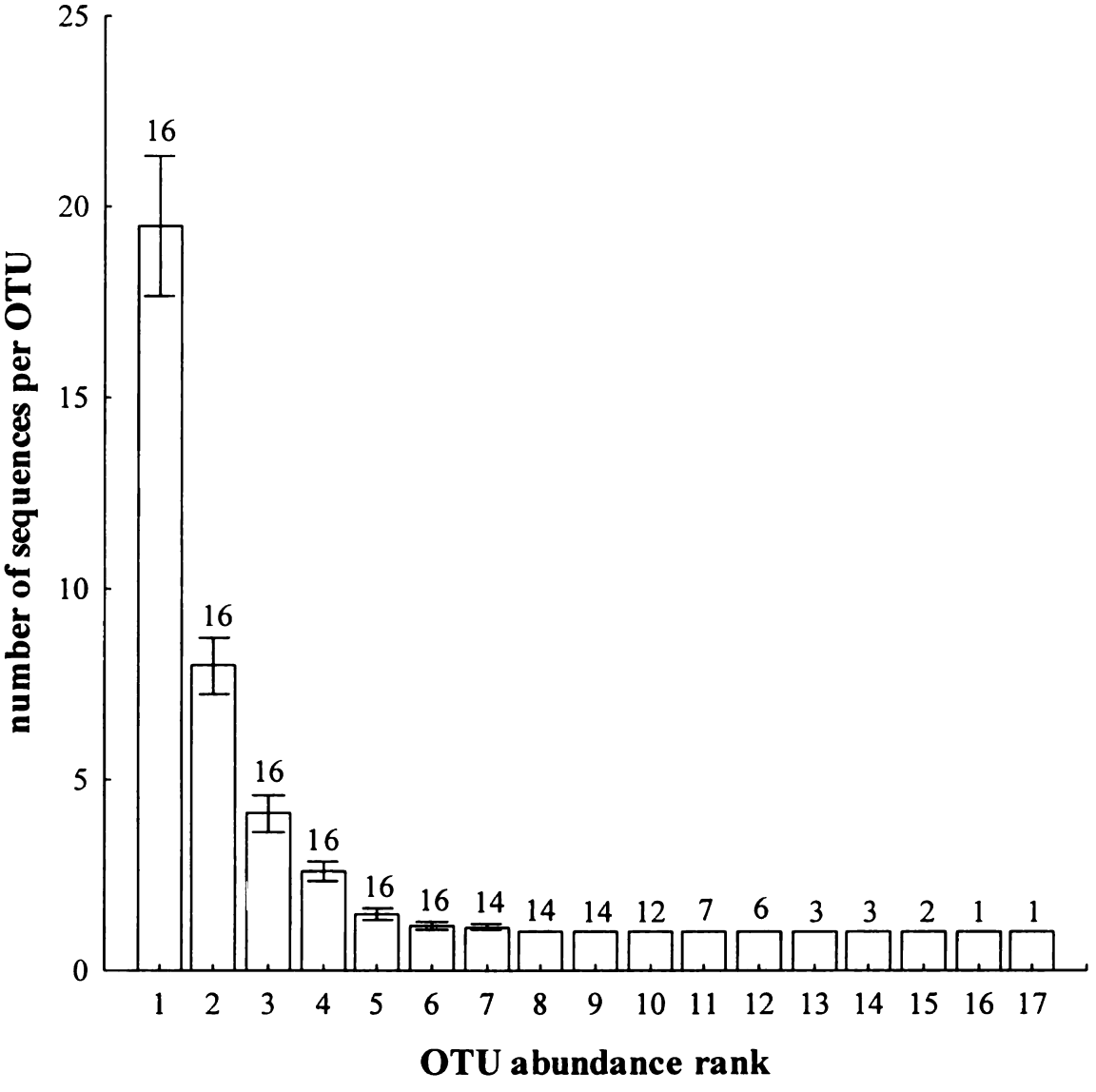



Figure 4.6. Rank abundance curve showing the relative abundance of OTUs within the anal pouch of each sampled hyena. The sample sizes indicate the number of libraries that contained at least that number of OTUs. Bars indicate standard errors.

sequence identity cutoff, our sampling captured the prominent members of these communities, so evaluating similarities in bacterial community structure among the anal pouches of individual hyenas was appropriate.

**Beta diversity**

An effect of clan membership on the structure of bacterial communities in the anal pouches of hyenas was evident in all three beta diversity analyses (ANOSIM global $R = 0.35$, $P = 0.001$; Figure 4.7; Figure 4.8). Although the samples obtained from Emarti Hill hyenas did not differ significantly in structure from those obtained from Mara River ($R = -0.01$, $P = 0.568$), Southern Comfort ($R = 0.40$, $P = 0.113$), or Fig Tree ($R = 0.19$, $P = 0.087$) hyenas, samples from the Mara River, Southern Comfort and Fig Tree hyenas clearly clustered with other samples from their respective clans (MR vs. SC: $R = 0.78$, $P = 0.028$; MR vs. FT: $R = 0.33$, $P = 0.030$; SC vs. FT: $R = 0.40$, $P = 0.028$). The high R-value between the Mara River and Southern Comfort clans indicated that community structure differed substantially among samples from these two clans. The R-values between Fig Tree and both Mara River and Southern Comfort clans were more moderate, indicating greater degrees of overlap in community structure. These patterns were evident in both visual analyses as well, as the samples from Fig Tree hyenas clustered less tightly than did those from Mara River and Southern Comfort hyenas.

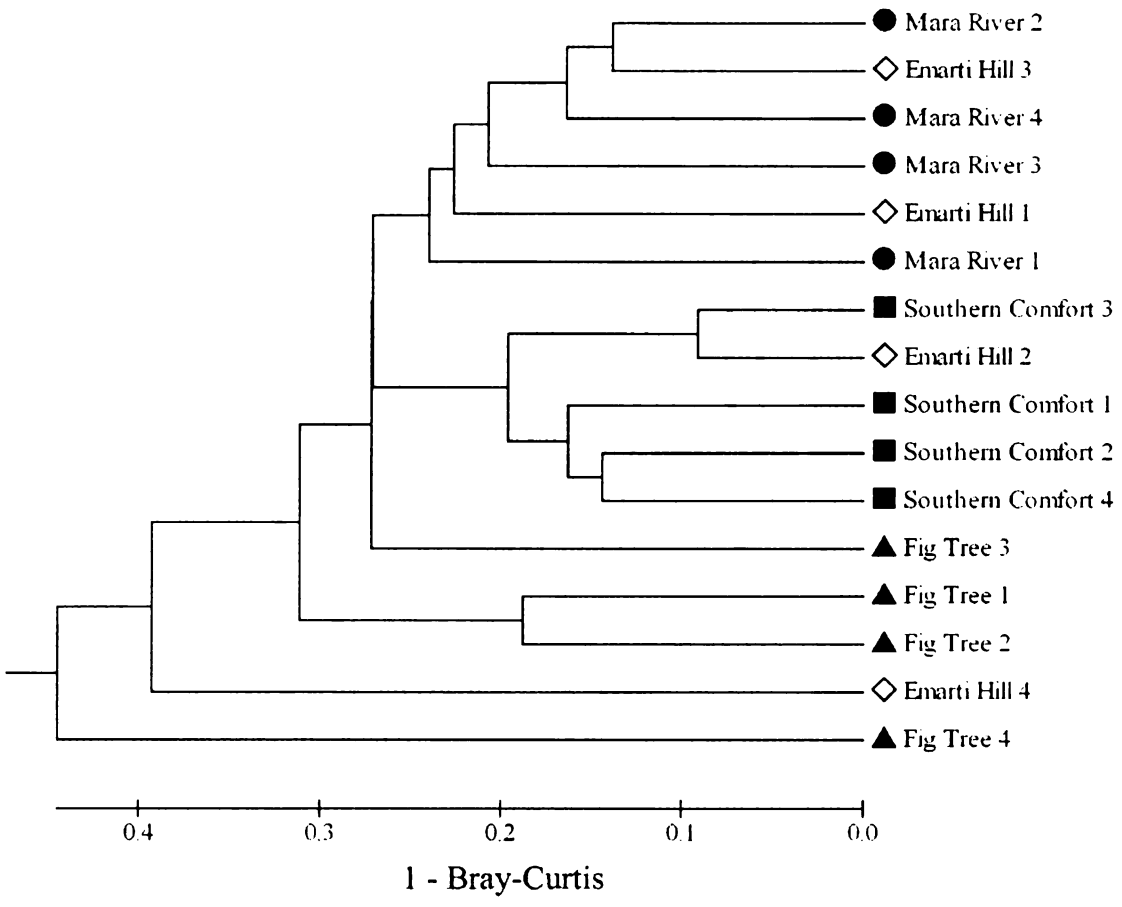



Figure 4.7. Dendrogram illustrating similarities in the structure of bacterial communities inhabiting the anal pouches of 16 spotted hyenas representing four distinct clans: Mara River (circles), Southern Comfort (squares), Emarti Hill (diamonds) and Fig Tree (triangles).

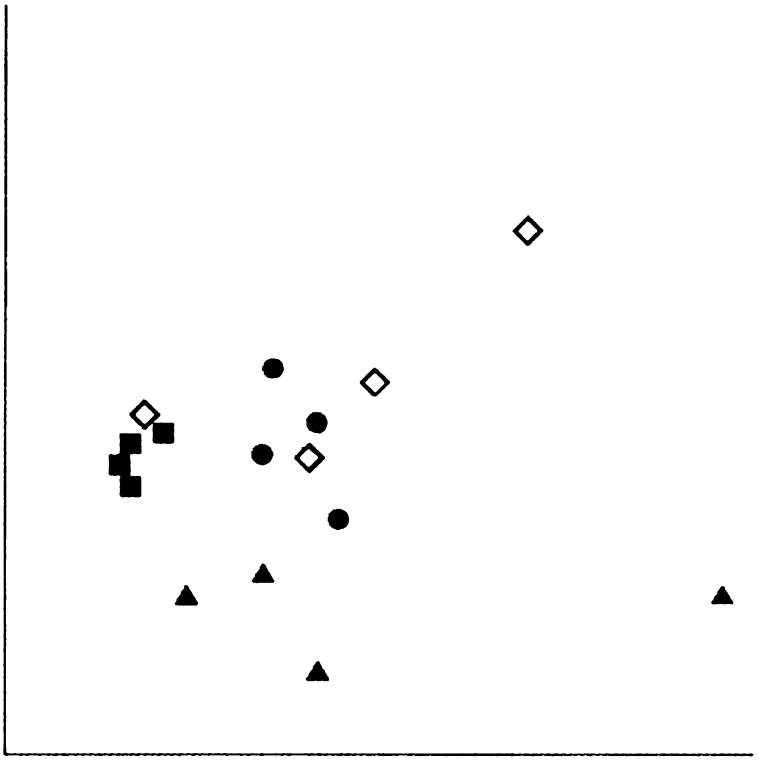


Figure 4.8. Multidimensional scaling (MDS) plot comparing similarities in the structure of bacterial communities inhabiting the anal pouches of 16 spotted hyenas representing four distinct clans: Mara River (circles), Southern Comfort (squares), Emarti Hill (diamonds) and Fig Tree (triangles). The plot was based on Bray-Curtis dissimilarity indices. In MDS plots, sampled communities that are dissimilar are placed far apart whereas those that are similar are placed close together (Quinn & Keough 2002; Gotelli & Ellison 2004). The stress of the plot is 0.123.

# DISCUSSION

## The microbiome of the spotted hyena anal pouch

In the current study, in which culture-independent microbial sampling techniques were employed, the vast majority (98%) of analyzed sequences were obtained from previously uncultured bacterial forms. Therefore, at the species-level, we were unable to determine the taxonomic identities of most members of the microbiome of the anal pouch of spotted hyenas. However, at an approximate genus-level, it was clear that the most prominent members of the anal pouch microbiome were *Peptostreptococcus*, *Anaerococcus*, *Propionibacterium*, and *Corynebacterium*. Previous culture-based surveys of the bacterial contents of the anal scent glands of carnivores yielded the following genera: *Streptococcus*, *Proteus*, *Bacillus*, *Eubacterium*, *Clostridium*, *Fusobacterium*, *Bacteroides*, *Peptococus*, and *Peptostreptococcus* (Indian mongoose, *Herpestes auropunctatus*: Gorman et al. 1974; red fox, *Vulpes vulpes*: Albone et al. 1978). The only genus found in both culture-based studies and in the current study was *Peptostreptococcus*. This finding reinforces the idea that culture-independent sampling techniques are valuable complements to culture-based approaches when investigating the structure and function of microbial communities (Riesenfeld et al. 2004).

*Peptostreptococcus* and *Anaerococcus* (both phylum *Firmicutes*) are anaerobic microbes that are frequently associated with the mouth, skin, and gastrointestinal and urinary tracts of mammals (Murdoch 1998; Ezaki et al. 2006). Importantly, peptostreptococci have been shown to produce several of the volatile fatty acid components of mammalian scent marks, including acetic, propionic, and butyric acids (Gorman et al. 1974). Each of these fatty acids has been identified in the paste of spotted

hyenas (Buglass et al. 1991; Hofer et al. 2001). *Propionibacterium* (phylum *Actinobacteria*) are predominantly anaerobic bacteria that comprise much of the mammalian skin microbiota, and are also symbionts within mammalian gastrointestinal and urinary tracts (Stackebrandt et al. 2006). Like peptostreptococci, propionibacteria have been shown to produce propionic and acetic acids as fermentation by-products (Stackebrandt et al. 2006; Cogen et al. 2008). *Corynebacterium* (phylum *Actinobacteria*) are aerobic or facultatively anaerobic microbes that are also often found in association with the skin surfaces and the gastrointestinal and urinary tracts of mammals (Liebl 2006; von Graevenitz & Bernard 2006). Corynebacteria have been shown to generate short and medium chain fatty acids when metabolizing large organic molecules associated with mammalian skin surfaces (James et al. 2004). In addition to the short chain fatty acids discussed above, numerous medium chain fatty acids have been identified in the paste of spotted hyenas (Buglass et al. 1991; Hofer et al. 2001). The fermentation hypothesis for the production of social odors among mammals suggests that volatile fatty acids are critical components of scent marks and that these fatty acids are by-products of bacterial fermentation within specialized scent glands (Albone et al. 1974; Gorman et al. 1974; Gorman 1976). Here we have provided evidence suggesting that bacteria capable of producing these fatty acids are present in the anal pouches of hyenas.

In addition to *Peptostreptococcus*, *Anaerococcus*, *Propionibacterium*, and *Corynebacterium*, our phylogenetic analyses also revealed the presence of *Finegoldia*, *Peptoniphilus*, *Cetobacterium*, *Ignavigranum*, *Pantoea*, *Pseudomonas*, *Cryptosporangium*, and *Segetibacter* bacteria in the anal pouch microbiome. *Finegoldia*, *Peptoniphilus*, *Cetobacterium*, and *Ignavigranum* are obligate or facultative anaerobic

bacteria that have been found previously in close association with mammalian bodies (Collins et al. 1999; Ruoff 2002; Finegold et al. 2003; Ezaki et al. 2006; Schaal et al. 2006). *Pantoea* and *Pseudomonas*, although notable plant pathogens (Pujol & Kado 2000; Kersters et al. 2006), have also been identified as mammalian symbionts (Rolph et al. 2001; Lim et al. 2006; Kersters et al. 2006). *Cryptosporangium* and *Segetibacter*, however, have not been identified as mammalian symbionts; rather, both genera are aerobic bacteria previously isolated from agricultural soils (Tamura et al. 1998; An et al. 2007). Presumably, these bacteria were picked up from the environment when hyenas pasted on grass stalks or when they lay on the ground. We do not know whether *Cryptosporangium* and *Segetibacter* have a symbiotic relationship with spotted hyenas or whether their occurrence in the survey was purely incidental.

**Similarities in the structure of bacterial communities within hyena clans**

Our analyses demonstrated similarities in the structure of bacterial communities within the anal pouches of spotted hyenas from the same clan. This was evident in the non-random clustering of samples from the same clan in both the dendrogram (Figure 4.7) and the MDS plot (Figure 4.8). It was also evident in the ANOSIM, as the null hypothesis was rejected that Bray-Curtis similarity indices were no greater within than between hyena clans. Substantial differences in the structure of bacterial communities within the anal pouches of Mara River and Southern Comfort hyenas were apparent. Samples from these two clans clustered tightly with other samples from their respective clans in both visual analyses, and the ANOSIM R-value was very high (0.78) between the Mara River and Southern Comfort clans, indicating that the bacterial communities of the

two clans were distinct. Differences in the structure of bacterial communities were also apparent between Fig Tree and Mara River hyenas, as well as between Fig Tree and Southern Comfort hyenas, but the degrees of difference were more moderate. This was because less similarity in bacterial community structure existed among members of the Fig Tree clan than among members of the Mara River or Southern Comfort clans. The primary reason for there being less similarity in community structure among members of the Fig Tree clan was that the anal pouch of one female (Fig Tree 4) was dominated by a single phylotype (35/46 sequences). The anal pouches of other members of the Fig Tree clan were more diverse, particularly with respect to evenness, as evidenced by the Shannon diversity indices within this clan.

Although similarities in bacterial community structure were evident within the Mara River, Southern Comfort and Fig Tree clans, there was little similarity in community structure among the anal pouches of Emarti Hill hyenas. Therefore, although our results showed that clan membership significantly affected the structure of bacterial communities within the anal pouches of spotted hyenas, clan membership was evidently not the only factor influencing community structure.

**Conclusions and future directions**

It has been postulated that symbiotic bacteria are responsible for the production of group-specific odors among social mammals that engage in scent marking (Albone et al. 1978). If symbiotic bacteria are responsible for the production of group-specific odors, then the structure of bacterial communities within the scent glands of social mammals should be more similar among group members than among members of different social

groups. Our analyses of the microbial contents of the anal pouches of free-living spotted hyenas affirm this prediction, providing support for the hypothesis that differences in bacteria are responsible for the production of group-specific odors in this species (Burgener et al. 2008; Theis et al. 2008). However, before accepting this hypothesis, additional predictions need to be tested. First, the specific bacterial strains in the anal pouch microbiome must generate the volatile fatty acids identified in paste. Although testing this prediction will be difficult, it can be accomplished using contemporary ecologically-guided approaches to cultivating previously uncultured anaerobic bacteria (Stevenson et al. 2004; Eichorst et al. 2007), coupled with gas chromatography / mass spectrometry analyses. Second, the bacterial profiles of paste samples must correlate with their chemical profiles (e.g. Xu et al. 2007). Third, hyenas must demonstrate that relative abundances of fatty acids alone provide a sufficient basis upon which to discriminate among samples. This prediction could be systematically tested via bioassays conducted with captive spotted hyenas housed at the Berkeley Field Station for the Study of Behavior, Ecology and Reproduction (University of California, Berkeley), and potentially with free-living spotted hyenas as well (see Chapter Three).

In the current study we broadly controlled for the age, sex and reproductive state of paste donors. In future studies, we intend to determine whether age, sex and reproductive state affect the structure of symbiotic bacterial communities in the scent glands of spotted hyenas, because these factors influence the structure of microbial communities associated with other mammalian hosts (age: Palmer et al. 2007; sex: Alexy et al. 2003; Voigt et al. 2005; reproductive state: Bartlett et al. 1977; but see Eschenbach et al. 2000; Coolen et al. 2005). Evaluating the effects of sex and reproductive state on

the symbiotic bacterial communities in the anal pouches of spotted hyenas would be particularly interesting given that information about both sex and female reproductive state are available to conspecifics in paste secretions (Chapter Three). Lastly, in the future, we intend to determine whether genotype (e.g. Newton et al. 2001; Lanyon et al. 2007), diet (e.g. Ley et al. 2008), or association patterns among hyenas within clans (e.g. Palmer et al. 2007), influence the structure of bacterial communities residing in the anal pouches of spotted hyenas. Our objectives in doing so are to ascertain the extent of the role bacteria play in the social lives of hyenas, while bringing together the all too often disparate fields of behavioral, microbial and chemical ecology.

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